Anemia and Erythropoietin in Heart Failure

P. van der Meer

The research presented in this thesis was supported by grant 920-03-219 of the NWO ZonMW to Peter van der Meer.

Financial support by the Netherlands Heart Foundation and Groningen University Institute for Drug Exploration (GUIDE) for the publication of this thesis is gratefully acknowledged.

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Van der Meer, Peter
Anemia and Erythropoietin in Heart Failure
Proefschrift Groningen. Met literatuuropgave en samenvatting in het Nederlands

ISBN 90-367-2406-6

Fotografie cover: Sipke Visser
Layout: Helga de Graaf i.s.m. Saar de Vries
Printed by: Ipskamp PrintPartners, Enschede The Netherlands.

RIJKSUNIVERSITEIT GRONINGEN

Anemia and Erythropoietin in Heart Failure

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 23 november 2005
om 16.15 uur

door

Peter van der Meer
geboren op 16 juli 1977
te Sneek

Promotores:	Prof. dr. D. J. van Veldhuisen Prof. dr. W. H. van Gilst
Copromotores:	Dr. A. A. Voors Dr. R.G. Schoemaker
Beoordelingscommissie:	Prof. dr. T.J. Rabelink Prof. dr. E.G. de Vries Prof. dr. F. Zijlstra

Paranimfen: Erik Lipsic
Jeroen Koning

The publication of this thesis was financially supported by Amgen BV, Roche Pharmaceutical, Novartis BV, Sanofi-Aventis BV, Bristol-Myers Squibb BV, Jansen-Cilag BV Ortho-Biotech, Astra Zeneca BV, GlaxoSmithKline, Merck Sharp & Dohme BV, Servier Nederland Farma BV, Pfizer, Bayer HealthCare, Boehringer Inghelheim, Sankyo Pharma, Menarini BV, Roche Diagnostics

Contents

General Introduction

Chapter 1

Introduction and aims of the thesis

General introduction and aims of this thesis

Introduction

The condition of Chronic Heart Failure (CHF) is currently a large public health problem. Patients with CHF have a high risk of death and cardiovascular complications and the prevalence of CHF is still increasing. The Framingham study demonstrated that the life time risk for developing CHF is 1 in 5 for both men and women(1). The etiology of CHF is diverse, but coronary artery disease leading to myocardial infarction is the main cause of CHF. Several other factors are involved in the etiology of CHF including hypertension, valvular disease, myocarditis and cardiomyopathy.

Pathophysiology of CHF

In CHF, independent of the aetiology, cardiac wall stress is increased and results in increased energy and oxygen demand. While myocardial perfusion at rest is often relatively normal, blood flow during exercise or after vasodilation is frequently impaired(2-4). The result of this apparent imbalance between oxygen demand and supply may play a key role in the pathophysiology of CHF. This has been observed, not only in patients with epicardial vascular disease but also in patients with idiopathic dilated cardiomyopathy (IDC), who have by definition anatomically normal coronary arteries(5-7). Van den Heuvel *et al*. showed that in patients with IDC, a decreased coronary blood flow reserve correlated with the severity of left ventricular dysfunction(3). Further evidence for the role of hypoxia in patients with IDC was provided by De Jong *et al*, who showed that 69% of the patients with IDC exhibited a decreased wall motion score during stress-testing(8). It is well known that ischemia may result in LV dysfunction, but these studies support the theory that the reverse may also be true.

Microvasculature and Neovascularization

Another contributory factor to the presence of hypoxia in CHF may be a deficient microvasculature, and in particular impaired new vessel formation (neovascularisation). In CHF, the vascular network has to support greater demands of the spared, hypertrophied myocardium. The relative insufficient vascularization might play a role in the pathophysiology of CHF. It follows that stimulation of neovascularisation may represent a useful therapeutic tool in patients with CHF(9). Hypoxia and ischemia are important for the upregulation of several pro-angiogenic factors, including Vascular Endothelial Growth Factor (VEGF) and Erythropoietin (EPO). Both proangiogenic factors have also been shown to be involved in the angiogenic response to myocardial ischemia(10;11). *In vitro*, stimulation of cultured endothelial cells with EPO resulted in proliferation and formation of vascular structures (12). Jaquet *et al*. compared the angiogenic properties of EPO with VEGF on endothelial cells derived from human myocardial tissue. They found that both proteins exhibited equal angiogenic properties indicating a possible pro-angiogenic effect (13). This was further studied in a rat model of stroke. EPO treatment, initiated 24 hours after occlusion of the middle cerebral artery, enhanced neovascularization and improved neurological function(14). Background on the effects of EPO on endothelial cells and neovascularization is further described in **chapter 2**.

Besides EPO, also VEGF levels may influence neovascularization in CHF and it is tempting to speculate this consequently influences outcome. Several polymorphisms are known in the VEGF gene and some are associated with alterations in VEGF production(15). Recently, we

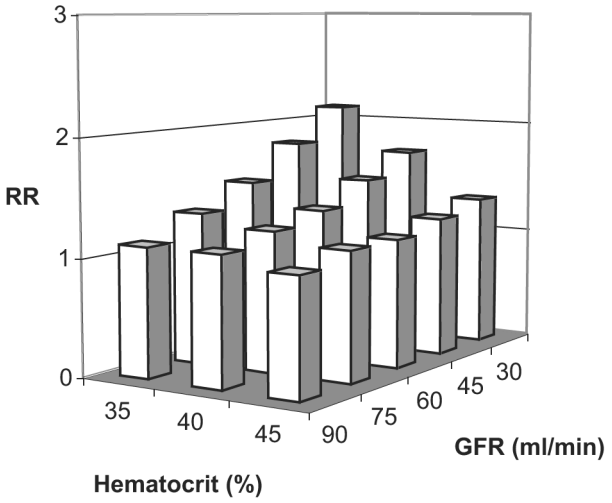


Figure 1. Standardized Relative Risk (RR) for all cause mortality predicted by level of GFR and hematocrit. Values are standardized to reference levels of hematocritof 45 and GFR of 90 ml/min.

investigated the effect of two common VEGF polymorphisms on morbidity and mortality in patients with Chronic Heart Failure, in a substudy of the MERIT-HF trial(16). We found that the presence of the VEGF polymorphism associated with lower VEGF levels was independently associated with an adverse outcome. A second VEGF polymorphism not associated with different VEGF levels, was not related to an altered prognosis. This study gives, although indirect, a clue that VEGF might be related to outcome in CHF patients.

Renal Function and Anemia

Reduced kidney function and anemia frequently occur in patients with CHF. Previous studies of our institution showed that as many as 25 to 50% of the CHF population has an estimated Gomerular Filtration Rate (GFRc) below 60 ml/min, indicating an impaired renal function. It has been demonstrated that renal function is a predictor of mortality in mild to advanced CHF patients(17-19). Hillege *et al.* studied renal function in advanced CHF patients and found patients in the lowest quartile of GFR (<44 ml/min) had a 3 times higher risk of mortality than patients in the highest quartile (>76 ml/min)(17). Several other studies found similar results regarding the prognostic value of kidney function in patients with an impaired left ventricular function(17-19).

In patients with end-stage renal disease anemia is a well-known risk factor for mortality(20). This was also shown to be true for patients with CHF. Several studies indicated that anemia is commonly observed in patients with CHF (21-23). One of the first studies published on anemia and CHF, showed in 142 patients with CHF, that mean hemoglobin (Hb) concentration decreased from 13.7 g/dl (8.6 mmol/L) in mild CHF to 10.9 g/dl (6.8 mmol/L) in severe CHF (24). As observed in patients with end-stage renal disease, anemia was not only common in CHF, but also associated with a decreased survival (22;23). One of the first studies published on this topic was performed by Horwich and colleagues, who found that hemoglobin levels < 12.3 g/dl (<7.7 mmol/L) were associated with worsened symptoms and an impaired survival. These findings were extended by the results of Al-Ahmad *et al.*, who showed that an impaired renal function and lower hematocrit levels were both independently

associated with higher mortality in CHF patients(25). In addition, in this study it appeared that both risk factors have a synergistic relationship, suggesting that anemia and reduction of kidney function are more than additive risk factors (Figure 1).

Erythropoietin

Almost hundred years ago two French scientists, Carnot and Deflandre, hypothesized that a circulating factor, hematopoietine, was responsible for the increase in red blood cells(26). They came to this theory by previous work from Monsieur Viault, who showed in 1890 that hemoglobin was higher in people living at high altitudes in the Andes(27). The name hematopoietine was replaced in 1948 by the term erythropoietin. After the purification of EPO in 1977, cloning was achieved and the subsequent production of recombinant erythropoietin lead to the first clinical trials in renal anemia(28). Several small trials in CHF showed beneficial effects of erythropoietin treatment in anemic CHF patients. EPO treatment resulted in an improved left ventricular ejection fraction, exercise capacity and in addition decreased the need for diuretics and hospital admissions(29;30).
Recent research indicates that EPO has pleiotropic effects well beyond the maintenance of red blood cells. The presence of the EPO-receptor outside the hematopoietic systems provides evidence for an autocrine and paracrine function(31;32).

Aims of this thesis

The **first part** of the current thesis studies the prevalence and origin of anemia in patients with CHF and the role of endogenous EPO herein. In **chapter 3** we study the origin of anemia in CHF patients. It has been shown in previous studies that only a minority of the anemic CHF patients suffers from hematinic deficiencies and severe renal failure, suggesting other factors to play a role in the development of anemia in CHF. This study demonstrates the effect of serum of anemic and non-anemic patients on hematopoietic progenitor cells and links the renin-angiotensin system with the occurrence of anemia in CHF. **Chapter 4** focuses on the predictive value for mortality of hemoglobin levels and plasma erythropoietin levels in CHF patients. In addition, it describes the correlation between plasma EPO levels and hemoglobin levels in CHF patients and controls.
The **second** part of the thesis emphasizes on the effects of EPO on cardiac function, beyond its classical hematopoietic effect. In **Chapter 5** the role of EPO and its receptor in the rat heart are studied in an ex-vivo ischemia-reperfusion model. In addition the acute effects of EPO on apoptosis are investigated. **Chapter 6 and 7** describe the results of chronic EPO treatment in rats with heart failure after myocardial infarction. In these chapters focus is put on the consequences of EPO on neovascularization. In **Chapter 8** several aspects of the acute and chronic effects of EPO on cardiac function are reviewed. **Chapter 9** summarizes this thesis and provides future perspectives on the origin and consequences of anemia in CHF and the results of EPO treatment in CHF beyond hematopoiesis.

References

1. Lloyd-Jones DM, Larson MG, Leip EP *et al*. Lifetime risk for developing congestive heart failure: the Framingham Heart Study. *Circulation*. 2002;106:3068-3072.

2. Neglia D, Michelassi C, Trivieri MG *et al*. Prognostic role of myocardial blood flow impairment in idiopathic left ventricular dysfunction. *Circulation*. 2002;105:186-193.

3. Van den Heuvel AF, van Veldhuisen DJ, van der Wall EE *et al*. Regional myocardial blood flow reserve impairment and metabolic changes suggesting myocardial ischemia in patients with idiopathic dilated cardiomyopathy. *J Am Coll Cardiol*. 2000;35:19-28.

4. Van den Heuvel AF, Bax JJ, Blanksma PK *et al*. Abnormalities in myocardial contractility, metabolism and perfusion reserve in non-stenotic coronary segments in heart failure patients. *Cardiovasc. Res*. 2002;55:97-103

5. De Boer RA, Pinto YM, van Veldhuisen DJ. The imbalance between oxygen demand and supply as a potential mechanism in the pathophysiology of heart failure: The role of microvascular growth and abnormalities. *Microcirculation*. 2003;10:113-126.

6. Liu PP, Mak S, Stewart DJ. Potential role of the microvasculature in progression of heart failure. *Am. J. Cardiol*. 1999;84:23L-26L.

7. Mela T, Meyer TE, Pape LA *et al*. Coronary arterial dimension-to-left ventricular mass ratio in idio pathic dilated cardiomyopathy. *Am. J. Cardiol*. 1999;83:1277-1280.

8. De Jong RM, Cornel JH, Crijns HJGM *et al*. Abnormal contractile responses during dobutamine stress echocardiography in patients with idiopathic dilated cardiomyopathy. *Eur. J. Heart Failure*. 2001;3:429-436.

9. Isner JM, Losordo DW. Therapeutic angiogenesis for heart failure. *Nat. Med*. 1999;5:491-492.

10. Shweiki D, Itin A, Soffer D *et al*. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*. 1992;359:843-845.

11. Zheng W, Brown MD, Brock TA *et al*. Bradycardia-induced coronary angiogenesis is dependent on vascular endothelial growth factor. *Circ. Res*. 1999;85:192-198.

12. Ribatti D, Presta M, Vacca A *et al*. Human erythropoietin induces a pro-angiogenic phenotype in cul tured endothelial cells and stimulates neovascularization *in vivo*. *Blood*. 1999;93:2627-2636.

13. Jaquet K, Krause K, Tawakol-Khodai M *et al*. Erythropoietin and VEGF exhibit equal angiogenic poten tial. *Microvasc Res*. 2002;64:326-333.

14. Wang L, Zhang ZG, Wang Y *et al*. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke*. 2004;35:1732-1737.

15. Watson CJ, Webb NJA, Bottomley MJ *et al*. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: Correlation with variation in VEGF protein production. *Cytokine*. 2000;12:1232-1235.

16. Van der Meer P, De Boer RA, White HL *et al*. The VEGF +405 CC Promoter Polymorphism is Associ ated With an Impaired Prognosis in Patients With Chronic Heart Failure: A MERIT-HF Substudy. *J Card Fail*. 2005;11:279-284.

17. Hillege HL, Girbes AR, de Kam PJ *et al*. Renal function, neurohormonal activation, and survival in patients with chronic heart failure. *Circulation*. 2000;102:203-210.

18. Dries DL, Exner DV, Domanski MJ *et al*. The prognostic implications of renal insufficiency in asymp tomatic and symptomatic patients with left ventricular systolic dysfunction. *J Am Coll Cardiol*. 2000;35:681-689.

19. Smilde TD, Hillege HL, Navis G *et al*. Impaired renal function in patients with ischemic and nonischemic chronic heart failure: association with neurohormonal activation and survival. *Am Heart J*. 2004;148:165-172.

20. Foley RN, Parfrey PS, Harnett JD *et al*. The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis*. 1996;28:53-61.

21. Cromie N, Lee C, Struthers AD. Anaemia in chronic heart failure: what is its frequency in the UK and its underlying causes? *Heart*. 2002;87:377-378.

22. Horwich TB, Fonarow GC, Hamilton MA *et al*. Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. *J Am Coll Cardiol*. 2002;39:1780-1786.

23. Mozaffarian D, Nye R, Levy WC. Anemia predicts mortality in severe heart failure: the prospective randomized amlodipine survival evaluation (PRAISE). *J Am Coll Cardiol*. 2003;41:1933-1939.

24. Silverberg DS, Wexler D, Blum M *et al*. The use of subcutaneous erythropoietin and intravenous iron for the treatment of the anemia of severe, resistant congestive heart failure improves cardiac and renal function and functional cardiac class, and markedly reduces hospitalizations. *J Am Coll Cardiol*. 2000;35:1737-1744.

25. Al-Ahmad A, Rand W.M, Manjunath G *et al*. Reduced kidney function and anemia as risk factors for mortality in patients with left ventricular dysfunction. *J Am Coll Cardiol*. 2003;38:955-962.

26. Carnot P, Deflandre C. Sur l'activite hematopoietique du serum au cours de la regeneration du sang. *C R Acad Sci (Paris)*. 1906;143:384-386.

27. Viault F. Sur l'augmentation considerable du nombre des globules rouge dans le sang chez les habi tants des hautes plateaux de l'Amerique du Sud. *C R Acad Sci (Paris)*. 1890;917-918.

28. Eschbach JW, Egrie JC, Downing MR *et al*. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. *N Engl J Med*. 1987;316:73-78.

29. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized controlled study. *J Am Coll Cardiol*. 2001;37:1775-1780.

30. Mancini DM, Katz SD, Lang CC *et al*. Effect of erythropoietin on exercise capacity in patients with

moderate to severe chronic heart failure. *Circulation*. 2003;107:294-299.

31. Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. *Early Hum Dev*. 1998;52:235-249.

32. Lappin TR, Maxwell AP, Johnston PG. EPO's alter ego: erythropoietin has multiple actions. *Stem Cells*. 2002;20:485-492.

Chapter 2

Erythropoietin in cardiovascular diseases

Peter van der Meer, Adriaan A. Voors, Erik Lipsic,
Wiek H. van Gilst, Dirk J. van Veldhuisen

a review

European Heart Journal 2004; 25: 285-291

Abstract

Several studies showed that anemia is commonly observed in patients with Chronic Heart Failure (CHF) and is associated with worsened symptoms and survival. When anemia in these patients is treated with erythropoietin (EPO), a significant improvement in cardiac function and symptoms was observed. Although it was originally believed that EPO specifically acted on hematopoietical cells, recent evidence demonstrated several non-hematopoietical effects. Ischemia/reperfusion experiments in rat heart and brain showed large infarct reduction when treated with EPO. Other effects of EPO are related to its pro-angiogenic effects on endothelial cells, which could be of potential value in patients with ischemic heart disease. These preclinical findings suggest that EPO may have potential effects in cardiovascular disease beyond correction of hemoglobin levels.

Introduction

In response to ischemia, mammalian cells express a variety of proteins, including hypoxia inducible factor 1α (HIF-1α). Expression of HIF- 1α increases exponentially, as cellular O_2 concentration decreases(1). Downstream effects of increasing levels of HIF-1α are upregulation of various proteins of which erythropoietin (EPO) plays a crucial role. EPO acts as a major regulator of erythropoiesis, by promoting the survival and proliferation of erythroid precursor cells(2). In response to hypoxia the kidney produces EPO, which in turn increases the number of red blood cells and thereby increasing the tissue oxygen supply.

Cloning of the human EPO gene was achieved in 1983. After the first clinical trials with recombinant human erythropoietin (rh-EPO), it has been used for more than a decade in the treatment of anemia in end stage renal failure(3). Rh-EPO has a direct effect on hematopoiesis, reflected by increased hemoglobin (Hb) levels. Decreased Hb levels are also common in patients with chronic heart failure (CHF) and although plasma EPO levels are increased in patients with CHF, they are still insufficient to counterbalance the decreased Hb levels(4). A recent study in anemic CHF patients showed that rh-EPO therapy significantly improved cardiac function and quality of life after correction of the Hb (5).

However, EPO can also exert non-erythropoietic effects. Recent evidence suggests that administration rh-EPO plays a protective role in vascular diseases(6-8). Ischemia/reperfusion experiments in rat heart and brain showed large infarct reduction when treated with EPO. The favorable effects of these EPO-related changes are still mostly unknown, but may be protection from apoptosis and its antioxidative properties (9;10). Other benefits of EPO in vascular disease and CHF may be related to its pro-angiogenic potentials(11). In this review, we discuss the current use of rh-EPO in cardiovascular diseases and its possible novel applications.

Renal failure, anemia and cardiac disease

Anemia is frequently observed in patients with chronic renal disease (CRF). When the definition of anemia, according to the World Health Organisation, is used (hemoglobin <12 g/dL (7.5 mmol/L) for women, <13 g/dl (8.1 mmol/L) for men and postmenopausal women),

over 80% of patients with a creatinine clearance < 25 ml/min are anemic(12). Renal anemia is an important risk factor for the development of cardiovascular disease(13). A study from Foley *et al*. showed that each 1 g/dl decrease in mean Hb in a renal failure population was independently associated with the presence of left ventricular dilatation and the development of de novo and recurrent cardiac failure(14). In addition, each 1g/dl decrease in mean Hb was also independently associated with mortality. Chronic anemia causes a long-lasting volume overload, which results in ventricular dilatation(15). Consequently, the length of the sarcomeres increases, leading to a better overlap between myofilaments. Furthermore, the thickness of the left ventricle increases to counterbalance the increased radius (eccentric hypertrophy). Left ventricular hypertrophy is an independent predictor of cardiovascular disease and significantly reduces life expectancy(16). A study in New Zealand also demonstrated that anemia together with hypertension and diabetes is one of the strongest independent predictors of left ventricular hypertrophy in patients with CRF(13).

EPO treatment in the CRF population

Since the first cloning and clinical testing of rh-EPO, fifteen years ago, the use of rh-EPO has become widespread in the treatment of (renal) anemia. More than a decade ago Wizemann *et al*. already showed that rh-EPO treatment in dialysis patients with significant coronary artery disease reduces exercise-induced myocardial ischemia, assessed by (ECG) treadmill test(17). Furthermore, besides the direct effect on myocardial oxygenation, correction of the anemia decreases cardiac output and cardiac workload, thus lowering oxygen consumption(18;19). Besarab *et al* studied 1233 hemodialysis patients with clinically evident ischemic heart disease or CHF(20). Patients were randomized to a normal hematocrit group (n=618), in which patients received doses of rh-EPO to achieve and maintain a hematocrit of 42%, and a low hematocrit group (n=615), in which patients received doses of rh-EPO sufficient to maintain a hematocrit of 30%. The primary end-point was time to death or a first non-fatal myocardial infarction. After 29 months, there were 183 deaths and 19 non-fatal myocardial infarctions among the normal-hematocrit group (42%), and 150 deaths and 14 non-fatal myocardials were observed among those in the low-hematocrit group (30%). Although patients in the normal-hematocrit group showed a trend towards increased mortality, it is important to note that higher rh-EPO doses or hematocrit values themselves were not associated with increased mortality. In fact, mortality rates decreased with increasing hematocrit values in each group.
A randomised cross-over study conducted in Australia investigated the effects of normalising Hb in comparison with sub-optimal Hb levels in haemodialysis patients treated with rh-EPO(21). Patients with higher Hb levels showed a significantly reduced Left Ventricular End-Diastolic Diameter (LVEDD). In addition, the incidence of LVH was significantly lower in patients with high Hb levels, compared to baseline. A Canadian multicenter trial failed to show that normalisation of Hb in patients with asymptomatic cardiomyopathy leads to regression of concentric LVH or LV dilatation(22). Nevertheless, treatment may have prevented additional LV dilatation. To evaluate the possible effect of rh-EPO therapy on the prevention of LVH, Hayashi *et al* studied the effects of rh-EPO in pre-dialysis patients. In patients with partially corrected anemia (Ht 30%) they observed a trend towards a reduction of LVH, whereas in patients with normalised Ht (Ht 40%) this decrease appeared to be statistically significant(23).
Taken together, in the later phases of cardiac disease in patients with CRF, when severe LVH

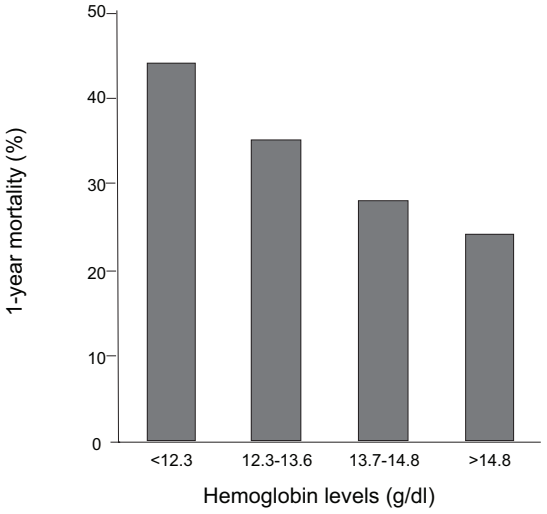


Figure 1. One-year mortality in NYHA functional class III and IV patients for the different hemoglobin levels (26).

or LV dilatation has developed, the effects of correction of anemia seem to be limited. On the other hand, these studies indicate that rh-EPO treatment has at least a favourable effect on the prevention of LVH, although the numbers of patients in each trial are low. An explanation for its limited effects in advanced heart disease could be the long-lasting development of LVH. The accompanied structural abnormalities of the heart, such as interstitial fibrosis (24) can lead to irreversible changes of ventricular structure and therefore may reduce the effects of treatment with rh-EPO.

Anemia and Chronic Heart Failure

Anemia is also commonly observed in patients with CHF and is related to the severity of disease. In 142 patients with CHF, mean Hb concentration decreased from 13.7 g/dl (8.6 mmol/l) in mild CHF (New York Heart Association [NYHA] class I) to 10.9 g/dl (6.8 mmol/l) in severe CHF (NYHA IV)(25). Furthermore, it has been shown that anemia has a prognostic value(26). Evidence for a relation between anemia and cardiac morbidity and mortality was provided by Al-Ahmad *et al.*(27). They retrospectively examined the Studies Of Left Ventricular Dysfunction (SOLVD) database (n=6.635) and found in univariate analysis that a reduced kidney function and lower hematocrit were both a risk factor for all-cause mortality. After adjusting for traditional cardiovascular risk markers they found that a 1% lower hematocrit was associated with a 1.027 higher risk for all-cause mortality. Similar findings were described by McClellan *et al*(28). They studied 665 randomly selected patients with the primary diagnosis CHF and also found in this population that both hematocrit and serum creatinine were independently associated with increased risk of death. Further, Horwich *et al*. studied the relation between anemia and mortality in a prospective cohort study in over 1000 patients with advanced heart failure (NYHA class III or IV) (26). They conclude that even mild degrees of anemia, Hb < 12.3 g/dl (7.7 mmol/l), are associated with an impaired

survival, and with worsened symptoms and functional status (figure 1). More evidence that anemia is associated with in impaired survival was provided by Ezekowitz *et al.*(29). They studied a large community-based cohort of patients (n=12.065) and found that anemia is observed in 17% of the patients and an independent prognostic marker for mortality. To determine the prevalence of anemia the authors used the International Classification of Diseases. In using this method, they could not provide a cutoff value for when they considered patients to be anemic. Therefore, this study may underestimate the prevalence of anemia in community-based patients with CHF(30).

Several factors play a role in the pathogenesis of anemia observed in CHF patients. It is known that anemia in chronic inflammatory diseases is associated with increased levels of cytokines, like Tumor Necrosis Factor (TNF) alpha. Recent studies showed that these cytokines play an important role in heart failure (31-33). It has been observed that patients with CHF express elevated levels of TNF-alpha, which in turn partly inhibits the hematopoiesis, a mechanism similar to other chronic diseases associated with anemia. Furthermore, malnutrition could play a role in the development of anemia in CHF. Horwich *et al.* showed that CHF patients with lower Hb levels were characterised by lower levels of albumin and a lower body mass index(26). The use of angiotensin converting enzyme (ACE) inhibitors, widely used in the CHF population, may also reduce the effects of EPO on erythropoiesis(34) and may result in anemia(35). In addition, many CHF patients use anti-coagulants, and chronic (microscopic) blood loss may well play a role. Furthermore, anemia can originate from reduced red blood cell (RBC) volume, but may also result from increased plasma volume (hemodilution). Androne *et al* evaluated these two origins of anemia in patients with CHF and found that hemodilution was observed in 46% of the patients with CHF referred for heart transplantation(36). In these two cohorts patients with hemodilution tended to have a higher mortality rate than anemia associated with a reduced RBC volume (p=0.08). These results indicate that treatment should not only be focussed on elevating RBC volume, but also on adjusting the dosage of diuretics in many anemic patients with severe CHF.

On the other hand, anemia can also provoke or worsen CHF. The interaction between anemia and CHF is complex, but there is expanding evidence that CHF can possibly exert anemia. Silverberg *et al.* recently proposed the so-called 'Cardio-Renal Anemia Syndrome', which provides a simple explanation for this phenomenon, where CHF can cause CRF and both can then cause anemia (34)(figure 2). Once anemia has developed, the increased cardiac workload results in LVH and ultimately worsens the cardiac function, which in turn worsens renal function, completing the vicious circle.

The role of rh-EPO in CHF

A recent study of Silverberg *et al.* showed the beneficial effects of rh-EPO therapy in CHF patients(5). They conducted a study in mild anemic patients with severe CHF (NYHA ≥III) and treated them with rh-EPO and intravenous iron. Thirty-two patients who had a left ventricular ejection fraction (LVEF) ≤ 40% and whose Hb levels were persistently between 10.0 and 11.5 g/dl (6.3-7.2 mmol/l) were randomized into two groups. The treatment group (16 patients) received rh-EPO and intravenous iron to increase the Hb from a mean of 10.3 g/dl to 12.9 g/dl (6.4 to 8.1 mmol/l). Over a mean of 8.2 +/- 2.6 months, the treated group showed an improvement of 42% in NYHA class, while NYHA class in the control

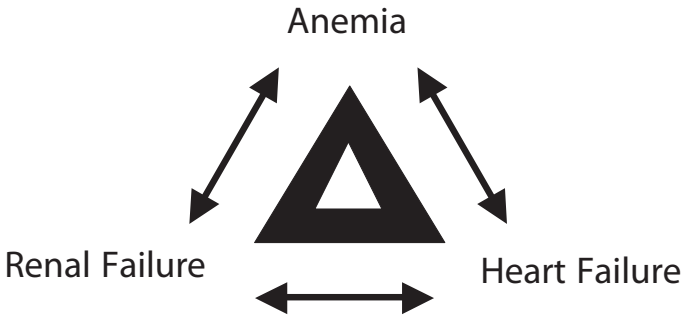


Figure 2 The cardio-renal anemia syndrome (34)

group worsened with 11%. Furthermore, left ventricular ejection fraction increased 5.5% in the treatment group, compared to a decrease of 5.4% in the control group. The number of hospitalisation days decreased by 79% in the treatment group and increased by 57.6% in the control group, compared to the same period before entering the study. Despite the small sample size (n = 32) and the open label design of this study, these results strongly suggest an important role for rh-EPO in the correction of even mild anemia in CHF. Mancini *et al.* studied the effects of rh-EPO on exercise capacity in patients with moderate to severe CHF(37). Twenty-six anemic patients with a mean Hb 11.0 g/dL (6.9mmol/L) were randomised to receive either EPO (15.000 to 30.000 IU per week) or placebo for three months. They found that EPO significantly enhanced exercise capacity, assessed by peak oxygen consumption and increased exercise duration, in patients with CHF. Even more important, quality of life was also significantly improved in the EPO treated group. Recently Silverberg and colleagues conducted a trial to study the effects of EPO treatment in diabetics and non-diabetics with severe CHF and mild to moderate renal failure(38). They found that correction of mild anemia (Hb 9.5-11.5 g/dL, [5.9-7.2 mmol/L]) in diabetics and non-diabetics resulted in an improved cardiac function, a better quality of life and a reduction in the number of hospitalisations. Although this was not a randomised placebo-controlled study, the results are comparable with previous studies and add to the expanding literature that diabetics benefit as much as non-diabetics from EPO treatment.

Anti-apoptotic effects of EPO

The previously described beneficial effects of rh-EPO on left ventricular structure and function are mainly explained by its effects on erythropoiesis. However, expanding evidence suggests that EPO plays a major role in non-erythropoietic processes. Several reports showed its efficacy in brain and retinal diseases, mainly by preventing apoptosis(9;39;40). As apoptosis has been implicated as a mechanism that contributes to the loss of cardiomyocytes in CHF(40) and ischemic injury(41), rh-EPO may have beneficial effects on these diseases as well.

Most of the anti-apoptotic effects of EPO are known from the hematological field. EPO is the primary regulator of erythropoiesis, and promotes the proliferation and differentiation of erythroid progenitor cells(2). It does so, at least partially, by preventing immature

erythroblasts from apoptotic cell death. EPO binds to a specific transmembrane receptor: the EPO-receptor (EPO-R). After binding with EPO, various signalling pathways are simultaneously activated, including the MAPK p42/44, JAK2-STAT5 and the PI-3-AKT proteins. Studies performed on immortalised human cell lines suggest that cell proliferation is regulated mainly by activation of MAPK p42/44 or JAK2-STAT5 and inhibition of apoptosis mostly by activation of the PI-3K-AKT axis or JAK2-STAT5(42). In the latter case, phosphorylated Jak2 triggers the activation of STAT5 protein and the activated STAT5 translocates into the nucleus, where it binds to specific DNA response elements and induces a cascade of cellular responses, including the upregulation of the anti-apoptotic genes such as bcl-2 and bcl-XL (43;44). This anti-apoptotic mechanism is not only important in erythropoiesis, but also appears to play an important role in other processes with high apoptotic activity, for example in stroke, retinal diseases and possibly myocardial infarction and CHF(7;40;41;45).

Effects of EPO on angiogenesis

Beside its anti-apoptotic effects, other non-erythropoietic effects of EPO have been described. A study of Juul *et al* revealed the presence of EPO and EPO-R in human fetal tissue. Although the heart showed minor amounts of EPO, the EPO-R was abundantly expressed in the myocardium as gestation progressed, indicating its presence in adult cardiac tissue(46). Recently we found that EPO-R is also expressed in the adult heart, including endothelial cells, fibroblasts and to a lesser extent on cardiomyocytes(47). Experiments with mice deleted (knocked-out) for the gene expressing EPO and EPO-R provided more evidence for its role in cardiac tissue as both $EPO^{-/-}$ and $EPO\text{-}R^{-/-}$ mice suffer from ventricular hypoplasia(48). This defect appears to be independent from the hypoxic state and is likely due to a reduction in the number of proliferating cardiac myocytes in the ventricular myocardium. To support this concept, additional experiments were performed under cultured conditions. In these experiments, Wu *et al.* found that EPO acts as a mitogen in isolated cardiomyocytes from $EPO^{-/-}$ and wild type mice, while it has no effect in $EPO\text{-}R^{-/-}$ mice(48). These findings together strongly suggest that EPO and its receptor, at least during fetal life, stimulate cardiomyocyte proliferation.

Another interesting observation in the experiments with EPO knockout mice was that the vascular network in the mutant rodent was also severely affected, with a disorganised structure. Instead of inter-connected, fine vascular networks, the $EPO\text{-}R^{-/-}$ heart showed dilated and independent vascular clumps(48). Indirect evidence suggests that subtotally nephrectomisd rats with moderate renal failure, and presumably low levels of EPO, showed a lack of microvessels in the heart(49). The expression of EPO-R has been demonstrated on endothelial cells *in vivo* and *in vitro*(50). Stimulation of cultured endothelial cells with rh-EPO resulted in cell proliferation and differentiation into vascular structures(11;51) and incubation of rat aortic rings with rh-EPO was related to endothelial sprouting(52). Recently, Jaquet *et al.* compared the angiogenic potentials of EPO with VEGF on endothelial cells derived from the myocardium(53). They found that both proteins exhibit equal angiogenic potentials, implying a role of rh-EPO in vasoproliferative processes.

Downstream effects of increasing levels of HIF-1α are upregulation of EPO and VEGF, reviewed by Jelkmann *et al.* (54). It is already known that the latter one causes angiogenesis, which has also been documented in the myocardium. Recent work of our group showed that VEGF might play a role in heart failure, particularly in idiopathic dilated cardiomyopathy

(IDC) (55). We observed a decreased capillarisation in IDC, which is disproportionate to the rate of hypertrophy and may contribute to the demand-supply mismatch (56). The reason for this seemingly decreased angiogenic capacity could be the reduced expression of VEGF in IDC. On the other hand, CHF is also associated with an increase in apoptosis (40;57). Both findings in CHF, decreased capillarisation and increased apoptosis could possibly be influenced by rh-EPO due to its anti-apoptotic effects and through stimulation of angiogenesis.
Recently, endothelial progenitor cells (EPCs) have been isolated from adult peripheral blood(58). It has been shown that they are derived from the bone marrow, and play a role in physiological and pathophysiological neovascularisation(58;59). Heeschen *et al*. provided evidence that EPO stimulates neovascularisation, in part by enhancing EPO mobilisation from the bone marrow. In addition, they demonstrated, in a model of hind-limb ischemia, that EPO increased capillary density by 1.6-fold, compared with control mice. The pathophysiological relevance of these findings was further elucidated in patients with coronary heart disease. They found that patients with unstable coronary heart disease had significantly higher serum EPO levels, which were significantly associated with an increased amount of functionally active EPCs. Therefore, it is tempting to speculate that the dramatic results of EPO treatment in CHF(5) might, in part, be related to the mobilisation of EPCs.

The role of rh-EPO in stroke

One of the first reports on the effects of EPO and its receptor in brain was published in 1995 by Digicaylioglu *et al*(60). They detected functional expression of the EPO-R and a hypoxic upregulation of EPO in the brain. Further evidence for this concept was provided by Sakanaka *et al*.(7). In experiments with gerbils, EPO infusion into the lateral brain ventricle was related to neuronal protection against ischemia-induced cell death. Specificity and biological relevance of these changes have been demonstrated by their observation that neutralisation of endogenous EPO with soluble EPO-R augments ischemic brain damage. The nature and mechanism of the protective role of EPO was further studied by Siren *et al* (9). They linked the prevention of ischemia-induced cell death to the anti-apoptotic effects of EPO. In rats, focal ischemia was induced by occlusion of the middle cerebral artery to produce an area of ischemia surrounded by a penumbra. In the latter area, programmed cell death is prominently present, and the anti-apoptotic effects of systemic rh-EPO therapy are impressive. Administration of EPO after 6 hours of arterial occlusion still provided a 50% reduction in infarct size, making rh-EPO a potential candidate for the treatment of ischemic diseases in humans. The encouraging findings in animal studies resulted in a double blind randomised proof-of-concept trial to investigate the safety and efficacy of rh-EPO for the treatment of ischemic stroke in man(61). Forty patients received either rh-EPO (33.000 U) or saline daily for 3 days after stroke. No adverse events were observed in the EPO treated groups. After one month, they observed an improvement in clinical outcome and a trend towards reduction in infarct size, assessed by MRI-scan, in the EPO treated patients (figure 3A). Furthermore, the serum marker of brain infarct damage (S100β) was identical in both groups on admission and increased after time of infarct, peaking at day 7. The rh-EPO group peaked at a lower level than the placebo group (figure 3B). However, larger trials are needed to confirm the trends observed in this study.

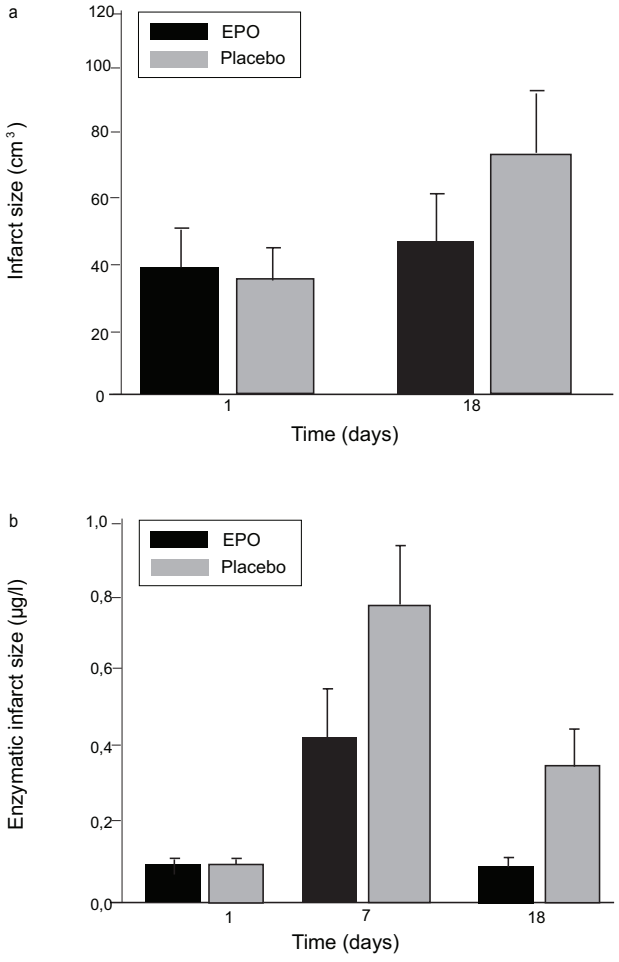


Figure 3. Evaluation of brain infarct size in patients treated with high dose rh-EPO or placebo for three consecutive days (16). Data represent mean +/- SEM. A. Infarct size measured by MRI on baseline and day 18. B. Time course of serum levels of S100β, a marker of brain damage.

The role of rh-EPO in ischemia and myocardial infarction

As there are many similarities between brain and heart ischemia, recent studies have been conducted to evaluate its possible effect in cardiac ischemia. Calvillo *et al*. assessed the potential protective role of EPO in vitro with adult rat cardiomyocytes, and *in vivo* in a rat model of myocardial infarction with reperfusion(8). They showed that EPO reduced the amount of apoptotic cells by 30% in cell culture and normalised hemodynamic function within one week after reperfusion *in vivo*. Recently we conducted ischemia/reperfusion experiments in the isolated rat heart to evaluate possible beneficial effects of EPO treatment (submitted). Administration of EPO reduced the cellular damage by 56% ($p<0.05$) and apoptosis by 15% ($p<0.05$) during reperfusion and resulted in a significantly improved recovery of left ventricular pressure ($p=0.02$) and coronary flow ($p=0.01$). Although the mechanism through

which EPO preserves cardiac function has yet to be elucidated, it is tempting to speculate that anti-apoptotic properties of EPO play a pivotal role. A recent paper from Scarabelli *et al* showed that in the early stages of reperfusion, apoptosis is first seen in endothelial cells and then spreads to surrounding cardiomyocytes, suggesting reperfusion induced release of pro-apoptotic mediators from endothelial cells(62). Although ischemia is able to initiate the apoptotic cascade, reperfusion is required to complete the apoptotic program (63). This is consistent with the findings that EPO limits cellular damage mainly during reperfusion, and to a lesser extent in the ischemic period. As the EPO-R in the heart is also expressed on endothelial cells, EPO treatment may prevent apoptosis in endothelial cells during reperfusion and thereby protect the myocardium and preserving vascular flow. Although these studies were all performed in rodents, apoptosis also plays an important role in patients with acute myocardial infarction. Saraste *et al*. observed apoptotic cardiomyocytes, particularly in the border zones of the infarcted myocardium, comparable to the penumbra in brain ischemia(41). As the apoptotic activity reaches its peak during the reperfusion period, beneficial effects of rh-EPO could possibly be achieved in patients receiving trombolytic therapy or after primary Percutaneous Coronary Intervention (PCI). Furthermore, it has been shown that EPO influences, at least during fetal life, cardiomyocyte proliferation. Therefore, it is interesting to speculate that infarct size on one hand could be limited through the anti-apoptotic effects of rh-EPO, but also could be influenced by increased proliferation.

Conclusion

Erythropoietin has been used in cardiovascular medicine for many years. Initially, research focussed on the direct effect on hematopoiesis and correction of anemia. Normalisation of the Hb level in mild anemic patients with CHF showed a positive effect on left ventricular function, a reduction in hospitalisation days and even more important an increase in quality of life. Although it was originally believed that EPO specifically acted on hematopoietical cells, recent evidence demonstrated several non-hematopoietical effects. The large reduction of infarction size after rh-EPO therapy in ischemia/reperfusion experiments in rat heart and brain are promising. As the EPO-R is present in cardiomyocytes and endothelial cells, one could speculate about its effects in other cardiac diseases. The anti-apoptotic effects might be specifically interesting in patients with CHF and myocardial infarction. The pro-angiogenic effects of EPO, as effective as VEGF, are also of potential value in patients with ischemic heart disease. Taken together, the future of rh-EPO therapy in cardiovascular diseases seems promising. Further basic research into the molecular mechanisms of the apoptosis cascade in cardiomyocytes and pro-angiogenic effects on EPCs and endothelial cells will be necessary to delineate the benefits of rh-EPO therapy in cardiovascular medicine. Finally, randomised clinical trials have to prove if its theoretical advantages will be translated into clinical practice.

References

1. Wang GL, Jiang BH, Rue EA, *et al*. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodi mer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-4.

2. Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev 1992;72: 449-89.

3. Bommer J, Kugel M, Schoeppe W *et al*. Dose-related effects of recombinant human erythropoietin on erythropoiesis. Results of a multicenter trial in patients with end- stage renal disease. Contrib Nephrol 1988;66:85-93.

4. Volpe M, Tritto C, Testa U *et al*. Blood levels of erythropoietin in congestive heart failure and correlation with clinical, hemodynamic, and hormonal profiles. Am J Cardiol 1994;74:468-73.

5. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized controlled study. J Am Coll Cardiol 2001;37:1775-80.

6. Morishita E, Masuda S, Nagao M, *et al*. Erythropoietin receptor is expressed in rat hippocampal and cere bral cortical neurons, and erythropoietin prevents in vitro glutamate- induced neuronal death. Neuroscience 1997;76:105-16.

7. Sakanaka M, Wen TC, Matsuda S *et al*. *In vivo* evidence that erythropoietin protects neurons from isch emic damage. Proc Natl Acad Sci U S A 1998;95:4635-40.

8. Calvillo L, Latini R, Kajstura J *et al*. Recombinant human erythropoietin protects the myocardium from ischemia- reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci U S A 2003;100:4802-6.

9. Siren AL, Fratelli M, Brines M *et al*. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A 2001;98:4044-9.

10. Chattopadhyay A, Choudhury TD, Bandyopadhyay D, *et al*. Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. Biochem Pharmacol 2000;59:419-25.

11. Ribatti D, Presta M, Vacca A *et al*. Human erythropoietin induces a pro-angiogenic phenotype in cul tured endothelial cells and stimulates neovascularization *in vivo*. Blood 1999;93:2627-36.

12. Levin A, Thompson CR, Ethier J *et al*. Left ventricular mass index increase in early renal disease: Impact of decline in hemoglobin. Am J.Kidney Dis 1999;34:125-34.

13. Greaves SC, Gamble GD, Collins JF, *et al*.. Determinants of left ventricular hypertrophy and systolic dysfunction in chronic renal failure. Am J Kidney Dis 1994;24:768-76.

14. Foley RN, Parfrey PS, Harnett JD, *et al*. The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. Am J Kidney Dis 1996;28:53-61.

15. Anand IS, Chandrashekhar Y, Ferrari R, *et al*. Pathogenesis of Edema in Chronic Severe Anemia - Studies of Body-Water and Sodium, Renal-Function, Hemodynamic Variables, and Plasma Hormones. British Heart Journal 1993;70:357-62.

16. Parfrey PS, Foley RN, Harnett JD, *et al*. Outcome and risk factors for left ventricular disorders in chronic uraemia. Nephrol Dial Transplant 1996;11:1277-85.

17. Wizemann V, Kaufmann J, Kramer W. Effect of erythropoietin on ischemia tolerance in anemic hemo dialysis patients with confirmed coronary artery disease. Nephron 1992;62:161-5.

18. Fellner SK, Lang RM, Neumann A, *et al*. Cardiovascular Consequences of Correction of the Anemia of Renal-Failure with Erythropoietin. Kidney International 1993;44:1309-15.

19. Metivier F, Marchais SJ, Guerin AP, *et al*. Pathophysiology of anaemia: focus on the heart and blood ves sels. Nephrol Dial Transplant 2000;15 Suppl 3:14-8.

20. Besarab A, Bolton WK, Browne JK *et al*. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. N Engl J Med 1998;339:584-90.

21. McMahon LP, Mason K, Skinner SL, *et al*. Effects of haemoglobin normalization on quality of life and cardiovascular parameters in end-stage renal failure. Nephrol Dial Transplant 2000;15:1425-30.

22. Foley RN, Parfrey PS, Morgan J *et al*. Effect of hemoglobin levels in hemodialysis patients with asymp tomatic cardiomyopathy. Kidney Int 2000;58:1325-35.

23. Hayashi T, Suzuki A, Shoji T *et al*. Cardiovascular effect of normalizing the hematocrit level during erythropoietin therapy in predialysis patients with chronic renal failure. Am J Kidney Dis 2000;35:250-6.

24. Francis GS, McDonald KM, Cohn JN. Neurohumoral activation in preclinical heart failure. Remodeling and the potential for intervention. Circulation 1993;87:IV90-IV96.

25. Silverberg DS, Wexler D, Blum M *et al*. The use of subcutaneous erythropoietin and intravenous iron for the treatment of the anemia of severe, resistant congestive heart failure improves cardiac and renal function and functional cardiac class, and markedly reduces hospitalizations. J Am Coll Cardiol 2000;35:1737-44.

26. Horwich TB, Fonarow GC, Hamilton MA, *et al*. Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. J Am Coll Cardiol 2002;39:1780-6.

27. Al-Ahmad A, Rand W.M, Manjunath G *et al*. Reduced kidney function and anemia as risk factors for mortality in patients with left ventricular dysfunction. J Am Coll Cardiol 2003;38:955-62.

28. McClellan WM, Flanders WD, Langston RD, *et al*. Anemia and renal insufficiency are independent risk factors for death among patients with congestive heart failure admitted to community hospitals: A population-based study. Journal of the American Society of Nephrology 2002;13:1928-36.

29. Ezekowitz JA, McAlister FA, Armstrong PW. Anemia is common in heart failure and is associated with poor outcomes: insights from a cohort of 12 065 patients with new-onset heart failure. Circulation 2003;107:223-5.

30. Van der Meer P, van Gilst WH, van Veldhuisen DJ. Anemia and congestive heart failure. Circulation 2003;108:E41-E42.

31. Iversen PO, Woldbaek PR, Tonnessen T, *et al*. Decreased hematopoiesis in bone marrow of mice with congestive heart failure. Am J Physiol Regul Integr Comp Physiol 2002;282:R166-R172.

32. Torre-Amione G, Bozkurt B, Deswal A, *et al*. An overview of tumor necrosis factor alpha and the failing human heart. Curr Opin Cardiol 1999;14:206-10.

33. Rauchhaus M, Doehner W, Francis DP *et al*. Plasma cytokine parameters and mortality in patients with chronic heart failure. Circulation 2000;102:3060-7.

34. Silverberg DS, Wexler D, Iaina A. The importance of anemia and its correction in the management of severe congestive heart failure. Eur J Heart Fail 2002;4:681-6.

35. Macdougall IC. The role of ACE inhibitors and angiotensin II receptor blockers in the response to epoetin. Nephrol Dial Transplant 1999;14:1836-41.

36. Androne AS, Katz SD, Lund L *et al*. Hemodilution is common in patients with advanced heart failure. Circulation 2003;107:226-9.

37. Mancini DM, Katz SD, Lang CC, *et al*. Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. Circulation 2003;107:294-9.

38. Silverberg DS, Wexler D, Blum M *et al*. The effect of correction of anaemia in diabetics and non-diabetics with severe resistant congestive heart failure and chronic renal failure by subcutaneous erythropoietin and intravenous iron. Nephrol Dial Transplant 2003;18:141-6.

39. Brines ML, Ghezzi P, Keenan S *et al*. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 2000;97:10526-31.

40. Olivetti G, Abbi R, Quaini F *et al*. Apoptosis in the failing human heart. N Engl J Med 1997;336: 1131-41.

41. Saraste A, Pulkki K, Kallajoki M, *et al*. Apoptosis in human acute myocardial infarction. Circulation 1997;95:320-3.

42. Ratajczak J, Majka M, Kijowski J *et al*. Biological significance of MAPK, AKT and JAK-STAT protein acti vation by various erythropoietic factors in normal human early erythroid cells. Br J Haematol 2001; 115:194-204

43. Silva M, Grillot D, Benito A, *et al*. Erythropoietin can promote erythroid progenitor survival by repress ing apoptosis through Bcl-XL and Bcl-2. Blood 1996;88:1576-82.

44. Silva M, Benito A, Sanz C *et al*. Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. J Biol Chem 1999;274:22165-9.

45. Grimm C, Wenzel A, Groszer M *et al*. HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nat Med 2002;8:718-24.

46. Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. Early Hum Dev 1998;52:235-49.

47. Van der Meer P, Lipsic E, Boer RA, *et al*. A functional erythropoietin receptor in a rat heart is linked to anti-apoptotic effects. J Am Coll Cardiol 2003;41:330A.

48. Wu H, Lee SH, Gao J, Liu X, *et al*. Inactivation of erythropoietin leads to defects in cardiac morphogen esis. Development 1999;126:3597-605.

49. Amann K, Neimeier KA, Schwarz U *et al*. Rats with moderate renal failure show capillary deficit in heart but not skeletal muscle. Am J Kidney Dis 1997;30:382-8.

50. Anagnostou A, Liu Z, Steiner M *et al*. Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci U S A 1994;91:3974-8.

51. Ashley RA, Dubuque SH, Dvorak B, *et al*. Erythropoietin stimulates vasculogenesis in neonatal rat mes enteric microvascular endothelial cells. Pediatr Res 2002;51:472-8.

52. Carlini RG, Reyes AA, Rothstein M. Recombinant human erythropoietin stimulates angiogenesis in vitro. Kidney Int 1995;47:740-5.

53. Jaquet K, Krause K, Tawakol-Khodai M, *et al*. Erythropoietin and VEGF exhibit equal angiogenic poten tial. Microvasc Res 2002;64:326.

54. Jelkmann W, Hellwig-Burgel T. Biology of erythropoietin. Adv Exp Med Biol 2001;502:169-87.

55. De Boer RA, Henning RH, Tio RA *et al*. Identification of a specific pattern of downregulation in expres sion of isoforms of vascular endothelial growth factor in dilated cardiomyopathy. Heart 2002;88:412-4.

56. Van Veldhuisen DJ, van den Heuvel AF, Blanksma PK, *et al*. Ischemia and left ventricular dysfunction: a reciprocal relation? J Cardiovasc Pharmacol 1998;32 Suppl 1:S46-S51.

57. De Boer RA, van Veldhuisen DJ, van der WJ *et al*. Additional use of immunostaining for active caspase 3 and cleaved actin and PARP fragments to detect apoptosis in patients with chronic heart failure. J Card Fail 2000;6:330-7.

58. Shi Q, Rafii S, Wu MHD *et al*. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362-7.

59. Asahara T, Masuda H, Takahashi T *et al*. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circulation Research 1999;85:221-8.

60. Digicaylioglu M, Bichet S, Marti HH *et al*. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A 1995;92:3717-20.

61. Ehrenreich H, Hasselblatt M, Dembowski C *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2002;8:495-505.

62. Scarabelli T, Stephanou A, Rayment N *et al*. Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. Circulation 2001;104:253-6.

63. Webster KA, Discher DJ, Kaiser S, *et al*. Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. J Clin Invest 1999;104:239-52.

Part 1: Pathophysiology of Anemia

Chapter 3

Levels of hematopoiesis inhibitor N-acetyl-seryl-aspartyl-lysyl-proline partially explain the occurrence of anemia in heart failure

Peter van der Meer, Erik Lipsic, B. Daan Westenbrink, Ruud M.A. van de Wal, Edo Vellenga, Regien G.Schoemaker, Dirk J. van Veldhuisen, Adriaan A. Voors, Wiek H. van Gilst

Circulation 2005; 112:1743-1747

Abstract

Background: Anemia is common in patients with chronic heart failure (CHF) and is associated with a poorer prognosis. However, only a minority of patients with CHF has an impaired renal function or underlying hematinic deficiencies. It has been shown that inhibition of renin angiotensin system (RAS) is associated with the development of anemia. The aim of the present study was to determine possible mechanisms linking anemia to RAS activity in CHF patients.

Methods and Results: We initially evaluated 98 patients with advanced stable CHF, treated with ACE inhibitors (left ventricular ejection fraction 28±1%, age 69±1 years and 80% male), ten of whom had an unexplained anemia (normal hematinics and no renal failure). These 10 anemic patients were matched with 10 non-anemic patients based on age and left ventricular ejection fraction. Serum ACE activity was 73% lower in anemic CHF patients compared to non-anemic CHF patients ($p=0.018$). Moreover, serum of these patients inhibited in vitro the proliferation of bone marrow derived erythropoietic progenitor cells of healthy donors by 17% ($p=0.003$). Levels of the hematopoiesis inhibitor N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), which is almost exclusively degraded by ACE, were significantly higher in anemic CHF patients and clearly correlated to erythroid progenitor cell proliferation ($r=-0.64$, $p=0.001$).

Conclusions: Serum ACE activity is markedly lower in anemic CHF patients and serum of these patients inhibits hematopoiesis. The clear correlation between Ac-SDKP and proliferation of erythroid progenitor cells suggests an inhibitory role of Ac-SDKP on hematopoiesis in CHF patients, which may explain the observed anemia in patients treated with ACE inhibitors.

Background

Anemia is present in a substantial part of the chronic heart failure (CHF) population, ranging from 14-55%, depending on the definition of anemia and severity of disease.[1] Lower hemoglobin levels are also independently associated with an impaired prognosis[2-5], but the cause of anemia in CHF is often unknown. A recent study in the UK demonstrated that only a minority of CHF patients had renal impairment or underlying hematinic deficiencies.[6] This was confirmed by Witte *et al*, who showed that less than one third of the CHF patients were iron, folate or vitamin B12 deficient.[7] Therefore, other factors may be involved in the origin of anemia in CHF.

Since the early 1980's, it has been demonstrated that the use of angiotensin-converting enzyme (ACE) inhibitors is associated with lower hemoglobin levels.[8,9] Possible mechanisms might be related to changes in the renin angiotensin system (RAS). *In vitro,* angiotensin II stimulated the proliferation of erythroid-progenitor cells, an effect inhibited by angiotensin II receptor blockers.[10] Furthermore, it has been shown that N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a strong inhibitor of hematopoietic stem cells, was found to be hydrolyzed almost exclusively by ACE. Indeed, ACE inhibitors markedly increased Ac-SDKP levels by 5-6 fold, and may therefore reduce hematopoietic activity.[11]

The aim of the present study was to determine possible mechanisms linking anemia to RAS activity in CHF patients. Accordingly, we evaluated the different components of the RAS and studied the effect of serum of anemic CHF patients on the proliferation of erythroid progenitor cells and compared this to non-anemic CHF patients and healthy controls.

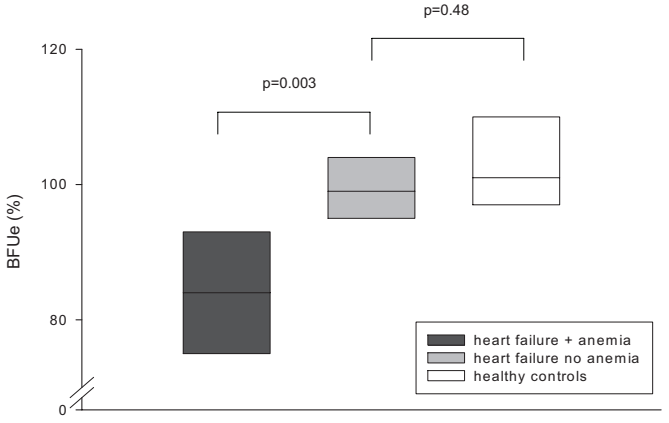


Figure 1 Comparison of the effect of serum on the formation of burst-forming units-erythroid (BFU-E), as a percentage of the baseline value (incubation without serum) in anemic, non-anemic and healthy controls. Boxplot show the median with 25-75% range of the BFU-E colonies present in culture.

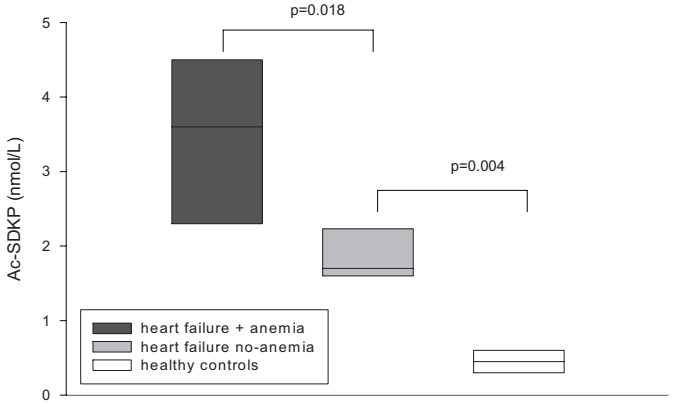


Figure 2 Levels of Ac-SDKP in anemic, non-anemic and healthy controls. Boxplot show the median with 25-75% range of Ac-SDKP in nmol/L.

Methods

Patients

In order to identify the patients with anemia of unknown origin, 98 consecutive patients with advanced systolic CHF were evaluated at our outpatient clinic, between February 2003, and November 2003. In all patients CHF was diagnosed on the basis of symptoms and the presence of left ventricular enlargement or systolic functional impairment by radionuclide ventriculography or echocardiography, according to the European Society of Cardiology Guidelines.[12] All patients had a left ventricular ejection fraction (LVEF) <45% and all patients were in New York Heart Association (NYHA) functional class III. Patients were stable on medication for at least three months and all patients were treated with an ACE inhibitor. The dose of the ACE inhibitor is expressed as a percentage of the recommended dose.[12] Patients using an angiotensin receptor blocker (ARB) or patients with myocardial infarction

Table 1. Baseline characteristics of the total population

Variable	Anemic (n=17)	Non anemic (n=81)	Total Population (n=98)	p-value
Hemoglobin levels (g/dl)	11.7 ± 0.2	13.9 ± 0.2	13.6 ± 0.2	-
Age (years)	72 ± 3	68 ± 1	69 ± 1	0.16
LVEF (%)	31 ± 3	28 ± 1	28 ± 1	0.33
Male sex (% male)	94	77	80	0.11
Ischemic etiology (%)	77	63	66	0.30
SBP (mmHg)	114 ± 3	125 ± 2	123 ± 2	0.02
NT-proBNP (pmol/L)	160 [80-231]	97 [33-244]	110 [49-239]	0.38
GFRc (ml/min)	54 ± 5	67 ± 3	64 ± 2	0.05
Serum Iron (µmol/L)	16.1 ± 1.3	16.0 ± 0.8	16.0 ± 0.7	0.94
Iron Saturation (%)	27.3 ± 3.4	23.8 ± 1.3	24.5 ± 1.2	0.26
Ferritin (µg/L)	111 ± 23	118 ± 10	116 ± 9	0.78
Folate (nmol/L)	12.2	12.0	12.0	0.90
Vitamin B12 (pmol/L)	261 ± 35	318 ± 19	307 ± 17	0.20
Medication (% use)				
ACE inhibitor	100	100	100	-
Beta Blocker	77	71	72	0.64
Diuretics	94	92	93	0.81
ACE genotype (%)				0.46
D/D+I/D	70.1	79.0	77.9	
I/I	29.4	21.0	22.1	

LVEF= left-ventricular ejection fraction. SBP= systolic blood pressure. NT-proBNP= N-terminal pro-brain natriuretic peptide. GFRc= estimated glomerular filtration rate. ACE= angiotensin-converting enzyme, p-value = anemic vs. non-anemic

(within 12 weeks), malignant or inflammatory diseases were excluded from this study.

To evaluate the hematopoietic response in patients without CHF, we included 8 healthy controls, without anemia and clinical signs of inflammation or CHF. The study was approved by the institutional review board and all patients provided written informed consent.

Hormonal measurements

Venous blood samples were taken at the outpatient clinic while the patient was in upright position. The blood samples were centrifuged and stored at −80°. Serum samples were thawed and analyzed immediately. High sensitive C-reactive protein (hs-CRP) was measured by nepholometry (BNII N; Nade Behring, Marburg, Germany), ACE activity by enzymatic assay (Bühlmann Laboratory AG, Schünenbuch, Switzerland), and TNF-α by immunoassay (R&D System, Minneapolis, USA). Ac-SDKP levels were determined by competitive enzyme immunoassay (Caymann, Ann Arbor, USA). In the plasma samples, N-Terminal pro-brain natriuretic peptide (NT-proBNP) was measured by immunoassay (Roche Diagnostics, Basel, Switzerland), erythropoietin (EPO) by immulite EPO assay (DPC, Los Angeles, USA) and an-

giotensin II levels by specific radioimmunoassay, as previously described.[13] All analyses were performed according to the manufacturer's guidelines.

Renal function and anemia

The glomerular filtration rate (GFR) is a standard indicator of renal function. Under steady-state conditions, GFR is estimated from serum creatinine using a formula that accounts for the influence of age and body weight on creatinine production, the Cockroft Gault equation: GFRc=[140-age in years)x(body weight in kg)]/(72xserum creatinine in mg/dL).[14] In women, the value is multiplied by 0.85. This formula is commonly used in several studies of CHF and renal function.[15,16] Anemia was defined according to the general accepted definition of the World Health Organization (hemoglobin ≤12 g/dL for women, ≤13 g/dl for men).

Hematopoiesis assay

Bone marrow cells of healthy donors were used for the in vitro burst-forming unit-erythroid (BFU-E) assay. Bone marrow mononuclear cells (BM-MNC) were isolated by Ficoll-Paque gradient centrifugation. Cells were collected, washed and resuspended in methylcellulose medium (Stemcell technologies Inc, Vancouver, Canada) with 2 U/ml recombinant-human EPO and 10% of serum of either anemic CHF patients, non-anemic CHF patients, healthy controls or without additional serum. All BFU-E assays were performed in quadruple. Blinded for sub-group allocation, BFU-Es were counted after 14 days of incubation. The number of BFU-E colonies in the absence of additional serum was considered to be the baseline value, and the number of BFU-E colonies was expressed as a percentage from baseline.

Genetic analysis

Genomic DNA was extracted from white blood cells. The ACE fragment containing the Insertion (I) / Deletion (D) sequence was detected by Polymerase Chain Reaction using oligonucleotide primers flanking the ALU insertion, as previously described.[17] Due to insufficient DNA extraction, in one patient no ACE I/D polymorphism could be determined.

Statistics

Data are given as mean ± SEM when normally distributed, as median and interquartile range (IQR) when skewed distributed and as frequencies for categorical variables. We compared differences between groups with the Student's t-test, Mann-Whitney U, or the ANOVA, with Fisher's protected LSD post-hoc analysis, where appropriate. Correlations between variables were expressed with the Pearson or Spearman correlation coefficient, where appropriate. We considered p-values below 0.05 as statistically significant. For all statistical analysis SPSS version 11.0 was used.

Results

Baseline characteristics of the total population are shown in table 1. The average age of the patients was 69±1 years, 80% were men, and the average LVEF was 28±1%. Mean hemoglobin levels were 13.6 g/dL (range 10.1-16.8 g/dL, median 13.6 g/dL). According to the WHO definition 17 of the 98 (17.3%) CHF patients were anemic. Anemia was associated

with lower calculated GFR (p=0.049) and lower systolic blood pressure (p=0.02), possibly reflecting hypoxia induced vasodilation. When log(EPO) was plotted as a function of hemoglobin, we observed only a weak non-significant correlation (r=-0.06, p=0.59).
We found that 4 of the 17 (24%) anemic CHF patients were vitamin B12 deficient [reference range: 170-750 pmol/L], one patient had low ferritin levels [reference range: 36-234 µg/L], and no folate acid deficiencies were observed [reference range: 4-30 nmol/L]. Two patients (12%) had a calculated GFR below 30 ml/min.
In the majority of anemic CHF patients (59%, n=10), no explanation for their anemia was found. We matched these 10 anemic patients with 10 non-anemic patients based on age and severity of heart failure (LVEF). Serum of anemic CHF patients inhibited the formation of BFU-Es by 17% compared to non-anemic CHF patients (p=0.003, figure 1). There was no difference in BFU-E formation between healthy controls and non-anemic CHF patients (p=0.48, figure 1). The anemic and non-anemic CHF patients were similar with regard to levels of angiotensin II, EPO, NT-proBNP, hs-CRP, TNF–α and GFRc, as well as duration and dose of ACE inhibitor use (table 2). However, ACE activity was 73% higher in the non-anemic, compared to anemic CHF patients (p=0.017; table 2). Consequently, Ac-SDKP levels were significantly higher in the anemic CHF patients compared to non-anemic CHF patients, whereas healthy controls had the lowest Ac-SDKP levels (figure 2). In addition, there was a strong correlation between Ac-SDKP levels and BFU-E formation (r=-0.64, p=0.001).
Regarding the ACE I/D polymorphism, patients homozygous for the I-allele were present only in the anemic sub-group (table 2, p=0.07). Furthermore, the ACE I/D polymorphism was related to BFU-E formation. Patients homozygous for the I-allele showed significant lower proliferation of erythroid progenitor cells compared to patients with DD or ID genotypes (p=0.046 and p=0.026, respectively).

Discussion

In the present study we show for the first time that CHF patients without identifiable cause of anemia have lower ACE activity and their serum inhibits the proliferation of bone marrow derived erythropoietic cells. We found that levels of Ac-SDKP, a strong hematopoiesis inhibitor, were significantly higher in these anemic CHF patients. Since ACE is the principle enzyme metabolizing Ac-SDKP, this links ACE activity to lower hemoglobin levels.
The decline of hemoglobin levels with ACE inhibitor use has been observed in numerous patients populations [18-21] as well as healthy volunteers.[22] Furthermore, ACE inhibitors are successfully used in the treatment of post-transplant polycythemia[18] and in subjects with altitude polycythemia.[20] These effects were also observed in CHF patients. A recent substudy of the Studies Of Left Ventricular Dysfunction (SOLVD), demonstrated convincingly that enalapril significantly increased the odds of developing anemia by 56% in patients with CHF.[23] The reduction in hematocrit levels occurs within several months after the start of enalapril and is sustained for at least several years. However, the observed effect on hematocrit seems to be modest and might only affect a selected population. In addition, ACE inhibitor use was associated with a better survival even after adjusting for episodes of anemia and in patients with prevalent anemia.[23]
Various mechanisms might play a role in the negative effects of ACE inhibitors on hematopoiesis. ACE inhibitor therapy may directly decrease the production of EPO in kidney,

Table 2. Baseline characteristics of the subpopulation

Variable	Anemia (n=10)	Non-anemia (n=10)	P-value
Hemoglobin levels (g/dl)	11.5 ± 0.3	14.9 ± 0.3	-
Age (years)	68 ± 3	70 ± 4	-
LVEF (%)	33 ± 4	33 ± 3	-
Male sex (n)	8	9	0.53
Ischemic etiology (n)	7	7	1.00
SBP (mmHg)	115 ± 4	140 ± 7	0.005
cGFR (ml/min)	61 ± 5	72 ± 9	0.32
Erythropoietin (mU/ml)	15.5 ± 2.7	19.0 ± 3.4	0.43
NT-proBNP (pmol/L)	83 [38-161]	96 [57-313]	0.55
Angiotensin II (pmol/L)	10.1 [4.2-23.4]	13.0 [4.3-54.9]	0.74
ACE activity (U/L)	13.7 [11.3-21.4]	23.8 [18.9-61.2]	0.017
hs-CRP (mg/L)	3.1 [1.8-7.3]	4.6 [1.1-11.0]	0.53
TNF-α (pg/mL)	1.5 [1.0-2.5]	2.0 [1.0-3.6]	0.52
Medication (% use)			
ACE inhibitor	100	100	-
Duration of ACEi use (months)	23 [8-46]	43 [8-46]	0.22
% of recommended dose ACEi	50 [44-100]	63 [25-100]	0.75
Beta Blocker	90	70	0.48
Diuretics	90	90	1.00
ACE genotype (%)			0.07
D/D + I/D	70	100	
I/I	30	0	

SBP= systolic blood pressure. LVEF= left-ventricular ejection fraction. NT-proBNP= N-terminal pro-brain natriuretic peptide. GFRc= estimated glomerular filtration rate. TNF= tumor necrosis factor. Hs-CRP= high-sensitivity C-reactive protein. ACE= angiotensin-converting enzyme, ACEi= angiotensin-converting enzyme inhibitor

probably by inhibiting angiotensin II formation.[24,25] Recently, Ac-SDKP a natural inhibitor of pluripotent hematopoietic stem cell proliferation has been found to be associated with ACE inhibitor therapy. Ac-SDKP is a tetrapeptide that reversibly prevents the recruitment of hematopoietic stem cells into S phase of the cell cyclus, by maintaining them in G0 phase.[26] This tetrapeptide was found to be degraded almost exclusively by ACE, which can be blocked by ACE inhibitors.[27] Already a single dose of the ACE inhibitor captopril resulted in a 5.5 fold increase in the levels of Ac-SDKP.[11] In our study we found that Ac-SDKP levels were two times higher in anemic CHF patients. This was associated with a marked lower ACE activity in anemic compared to non-anemic CHF patients. The observed differences in ACE activity and therefore Ac-SDKP levels might be related to the ACE I/D genotype. We observed a lower hematopoietic activity in CHF patients homozygous for the ACE I-allele, which is linked to lower ACE activity. This is in line with previous findings of Varagunam *et al*, who showed that dialysis patients with the ACE ID/II genotypes, required significant higher dosages of human

recombinant EPO for the treatment of their renal anemia, compared to the patients homozygous for the D-allele.[28]

Several studies found a clear correlation between renal function and Ac-SDKP levels.[29] Comte *et al* found that besides degradation by ACE, Ac-SDKP is partially eliminated in the kidney.[30] We found that GFRc was lower in anemic CHF patients compared to non anemic CHF patients, which might have influenced Ac-SDKP levels. The clinical relevance of Ac-SDKP levels was further explored by Le Meur *et al*, who studied the relation between Ac-SDKP levels and the weekly dose of recombinant human EPO for the treatment of renal anemia.[29] They observed that dialysis patients required significantly more recombinant human EPO when their Ac-SDKP levels were higher, indicating an inhibitory effect of Ac-SDKP on hematopoiesis.

A study of Mrug *et al* showed that angiotensin II levels stimulate the proliferation of hematopoietic cells through activation of the angiotensin II type I receptor, this effect was abolished by addition of an angiotensin-receptor blocker (ARB).[10] One may speculate that ACE inhibitors lower angiotensin II levels and therefore inhibit the hematopoietic proliferation. In our study we included only patients using ACE inhibitors, without concomitant use of angiotensin receptor blockers. Since there were no significant differences in angiotensin II levels between anemic and non-anemic CHF patients, our study does not suggest a major contribution of angiotensin II levels in the observed decreased hematopoietic activity in CHF patients. However, because patients receiving ARB were not included in our study we are unable to draw conclusions on the possible effect of angiotensin II on hematopoiesis in CHF patients. Studies in larger groups of patients, also comparing the effects of ACE inhibitor and ARB treatment, should address this issue.

Several limitations have to be acknowledged. Hemodilution might have influenced the degree of anemia, although all patients were stable on medication for at least three months. Inflammation in patients with CHF might lower hemoglobin levels and induce anemia.[31] Inflammatory parameters measured in our study were not different in anemic and non-anemic CHF patients. However other pro-inflammatory cytokines might play a role in the pathogenesis of anemia in CHF. Furthermore, due to the relatively small sample size one should be cautious interpreting the link between the ACE I/D polymorphism and hematopoiesis. Studies analyzing larger groups of patients should clarify the possible relationship between anemia and ACE genotype in CHF patients. Because of these limitations, we regard our study mainly as hypothesis generating.

In summary, ACE activity of CHF patients without an identifiable cause of anemia is significantly lower compared to non-anemic CHF patients, and serum of these patients inhibits hematopoiesis. The correlation between Ac-SDKP and proliferation of erythropoietic progenitor cells suggests an inhibitory role of Ac-SDKP in hematopoiesis, linking the renin angiotensin system to hematopoiesis in CHF patients.

Acknowledgments

Dr. Peter van der Meer is supported by NWO-ZonMW. Dr. Erik Lipsic is supported by GUIDE. The funding source had no role in study design, data collection, data interpretation, or writing the report.

References

1. Van der Meer P., Voors AA, Lipsic E, van Gilst WH, van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. *Eur Heart J* 2004;25:285-291.

2. Ezekowitz JA, McAlister FA, Armstrong PW. Anemia is common in heart failure and is associated with poor outcomes: insights from a cohort of 12 065 patients with new-onset heart failure. *Circulation* 2003;107:223-225.

3. Horwich TB, Fonarow GC, Hamilton MA, MacLellan WR, Borenstein J. Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. *J Am Coll Cardiol* 2002;39:1780-1786.

4. Mozaffarian D, Nye R, Levy WC. Anemia predicts mortality in severe heart failure: the prospective randomized amlodipine survival evaluation (PRAISE). *J Am Coll Cardiol* 2003;41:1933-1939.

5. Van der Meer P, Voors AA, Lipsic E, Smilde TDJ, van Gilst WH, van Veldhuisen DJ. Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure. *J Am Coll Cardiol* 2004;44:63-67.

6. Cromie N, Lee C, Struthers AD. Anaemia in chronic heart failure: what is its frequency in the UK and its underlying causes? *Heart* 2002;87:377-378.

7. Witte KK, Desilva R, Chattopadhyay S, Ghosh J, Cleland JG, Clark AL. Are hematinic deficiencies the cause of anemia in chronic heart failure? *Am Heart J* 2004;147:924-930.

8. Cleland JG, Dargie HJ, Hodsman GP, Ball SG, Robertson JI, Morton JJ, East BW, Robertson I, Murray GD, Gillen G. Captopril in heart failure. A double blind controlled trial. *Br Heart J* 1984;52:530-535.

9. Cleland JG, Gillen G, Dargie HJ. The effects of frusemide and angiotensin-converting enzyme inhibi tors and their combination on cardiac and renal haemodynamics in heart failure. *Eur Heart J* 1988;9:132-141.

10. Mrug M, Stopka T, Julian BA, Prchal JF, Prchal JT. Angiotensin II stimulates proliferation of normal early erythroid progenitors. *J Clin Invest* 1997;100:2310-2314.

11. Azizi M, Rousseau A, Ezan E, Guyene TT, Michelet S, Grognet JM, Lenfant M, Corvol P, Menard J. Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline. *J Clin Invest* 1996;97:839-844.

12. Remme WJ, Swedberg K. Guidelines for the diagnosis and treatment of chronic heart failure. *Eur Heart J* 2001;22:1527-1560.

13. Van den Meiracker AH, Admiraal PJ, Janssen JA, Kroodsma JM, de Ronde WA, Boomsma F, Sissmann J, Blankestijn PJ, Mulder PG, Man In 't Veld AJ, . Hemodynamic and biochemical effects of the AT1 receptor antagonist irbesartan in hypertension. *Hypertension* 1995;25:22-29.

14. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976;16:31-41.

15. Hillege HL, Girbes AR, de Kam PJ, Boomsma F, de Zeeuw D, Charlesworth A, Hampton JR, van Veld huisen DJ. Renal function, neurohormonal activation, and survival in patients with chronic heart failure. *Circulation* 2000;102:203-210.

16. Robertshaw M, Lai KN, Swaminathan R. Prediction of creatinine clearance from plasma creatinine: comparison of five formulae. *Br J Clin Pharmacol* 1989;28:275-280.

17. Rigat B, Hubert C, Corvol P, Soubrier F. Pcr Detection of the Insertion Deletion Polymorphism of the Human Angiotensin Converting Enzyme Gene (Dcp1) (Dipeptidyl Carboxypeptidase-1). *Nucleic Acids Research* 1992;20:1433.

18. Glicklich D, Kapoian T, Mian H, Gilman J, Tellis V, Croizat H. Effects of erythropoietin, angiotensin II, and angiotensin-converting enzyme inhibitor on erythroid precursors in patients with posttrans plantation erythrocytosis. *Transplantation* 1999;68:62-66.

19. Naito M, Kawashima A, Akiba T, Takanashi M, Nihei H. Effects of an angiotensin II receptor antagonist and angiotensin-converting enzyme inhibitors on burst forming units-erythroid in chronic hemodi alysis patients. *Am J Nephrol* 2003;23:287-293.

20. Plata R, Cornejo A, Arratia C, Anabaya A, Perna A, Dimitrov BD, Remuzzi G, Ruggenenti P. Angioten sin-converting-enzyme inhibition therapy in altitude polycythaemia: a prospective randomised trial. *Lancet* 2002;359:663-666.

21. Yildiz A, Cine N, Akkaya V, Sahin S, Ismailoglu V, Turk S, Bozfakioglu S, Sever MS. Comparison of the ef fects of enalapril and losartan on posttransplantation erythrocytosis in renal transplant recipients: prospective randomized study. *Transplantation* 2001;72:542-544.

22. Griffing GT, Melby JC. Enalapril (MK-421) and the white cell count and haematocrit. *Lancet* 1982;1:1361.

23. Ishani A, Weinhandl E, Zhao Z, Gilbertson DT, Collins AJ, Yusuf S, Herzog CA. Angiotensin-converting enzyme inhibitor as a risk factor for the development of anemia, and the impact of incident anemia on mortality in patients with left ventricular dysfunction. *J Am Coll Cardiol* 2005;45:391-399.

24. Chatterjee B, Nydegger UE, Mohacsi P. Serum erythropoietin in heart failure patients treated with ACE-inhibitors or AT(1) antagonists. *Eur J Heart Fail* 2000;2:393-398.

25. Pratt MC, Lewis-Barned NJ, Walker RJ, Bailey RR, Shand BI, Livesey J. Effect of angiotensin converting enzyme inhibitors on erythropoietin concentrations in healthy volunteers. *Br J Clin Pharmacol* 1992;34:363-365.

26. Lenfant M, Wdzieczak-Bakala J, Guittet E, Prome JC, Sotty D, Frindel E. Inhibitor of hematopoietic pluripotent stem cell proliferation: purification and determination of its structure. *Proc Natl Acad Sci*

U S A 1989;86:779-782.

27. Rousseau A, Michaud A, Chauvet MT, Lenfant M, Corvol P. The hemoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro is a natural and specific substrate of the N-terminal active site of human angiotensin-con verting enzyme. *J Biol Chem* 1995;270:3656-3661.

28. Varagunam M, McCloskey DJ, Sinnott PJ, Raftery MJ, Yaqoob MM. Angiotensin-converting enzyme gene polymorphism and erythropoietin requirement. *Perit Dial Int* 2003;23:111-115.

29. Le Meur Y, Lorgeot V, Comte L, Szelag JC, Aldigier JC, Leroux-Robert C, Praloran V. Plasma levels and metabolism of AcSDKP in patients with chronic renal failure: relationship with erythropoietin requirements. *Am J Kidney Dis* 2001;38:510-517.

30. Comte L, Lorgeot V, Volkov L, Allegraud A, Aldigier JC, Praloran V. Effects of the angiotensin-converting enzyme inhibitor enalapril on blood haematopoietic progenitors and acetyl-N-Ser-Asp-Lys-Pro concentrations. *Eur J Clin Invest* 1997;27:788-790.

31. Felker GM, Adams KF, Jr., Gattis WA, O'Connor CM. Anemia as a risk factor and therapeutic target in heart failure. *J Am Coll Cardiol* 2004;44:959-966.

Chapter 4

Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure

Peter van der Meer, Adriaan A. Voors, Erik Lipsic, Tom D. J. Smilde,
Wiek H. van Gilst, Dirk J. van Veldhuisen

Journal of the American College of Cardiology 2004; 44:63-67

Abstract

Objectives: This study aimed to investigate the prognostic importance of plasma erythropoietin (EPO) levels in Chronic Heart Failure (CHF) patients.
Background: Anemia is common and associated with an impaired survival in patients with CHF. EPO is a hematopoietic growth factor, upregulated in anemic conditions. Little is known about the pathophysiology of anemia in CHF and the prognostic importance of plasma EPO levels in CHF patients.
Methods: In 74 patients with CHF (age 61±2 years, Left Ventricular Ejection Fraction (LVEF) 0.31±0.01, peak oxygen consumption (VO_2) 19.1±0.6, (mean±SEM) and in 15 control patients, hemoglobin levels and plasma concentrations of EPO and BNP were measured.
Results: During a mean follow-up of 3.0 years (range 2.3-5.3), 22 patients (30%) died. Anemia was present in 24% of the patients. Multivariate analysis showed that plasma EPO (p=0.026) and hemoglobin levels (P=0.005) were independent predictors of survival in this CHF population. We observed only a mild inverse correlation between the logarithm of EPO, log(EPO), and hemoglobin levels (r^2=0.08, p = 0.02) in CHF patients, whereas the control group showed a clear significant inverse correlation (r^2=0.44, p=0.007).
Conclusion: Elevated plasma EPO levels are associated with an impaired prognosis independent of hemoglobin levels and other established markers of CHF severity. Furthermore, in the CHF patients, EPO levels poorly correlate with the Hb levels, in contrast to the control group

Introduction

Anemia is commonly observed in patients with CHF (1-3). One of the first studies published on anemia and CHF, showed in 142 patients with CHF, that mean hemoglobin (Hb) concentration decreased from 13.7 g/dl in mild CHF to 10.9 g/dl in severe CHF (4). Several other studies have also examined the prognosis of anemia in patients with CHF (2;3). Hb levels < 12.3 g/dl are associated with worsened symptoms, and impaired survival. Although large studies have confirmed these finding, the origin of anemia in CHF remains unclear (5).
In general, in response to anemia, the kidneys produce erythropoietin (EPO), which in turn stimulate red blood cell production (6;7). Previous studies already showed that patients with CHF exhibit elevated levels of EPO (8;9), although the significance of this finding has still to be elucidated. In addition, EPO levels may be correlated with the severity of CHF(8). Therefore, we hypothesized that elevated EPO levels are associated with a poorer prognosis. In the present study, we aimed to establish the prognostic value of both EPO and Hb on mortality in CHF patients.

Materials and methods

Patients

The prognostic values of EPO and Hb levels were retrospectively assessed in 74 Caucasian CHF patients with stable, mild to advanced CHF consecutively admitted to our center (a tertiary referral center), between 1998 and 2000. Referral of patients to our clinic was by general practitioners, cardiologists, local hospitals or other units of our hospital. Patients were treated for heart failure at the outpatient clinic. Patients were stable on medication for at least 3 months. In all patients, CHF was diagnosed on the basis of standard criteria, and presence of

left ventricular enlargement or systolic functional impairment by radionuclide ventriculography or echocardiography, according to the European Society of Cardiology guidelines(10). Patients with isolated diastolic dysfunction, valvular disease, myocardial infarction (within 12 weeks), cerebrovascular accident (within 12 weeks) or severe renal failure (creatinine >220 μmol/L) were excluded. To evaluate the EPO response in patients without CHF, we included 15 patients, referred to our center with complaints of chest pain or palpitations. Control patients had a mean age of 50.1±4.5 years and had a normal left ventricular function (LVEF ≥ 0.60), normal renal function, no clinical signs of inflammation and no evidence of CHF. All subjects gave informed consent for the protocol, which was approved by the local Medical Ethics Committee. Follow-up data were obtained by review of the medical record or telephone. The primary end point of the study was all-cause-mortality. No cardiac transplantations were observed in this population.

Measurement of BNP and EPO levels

Venous blood samples were taken in the morning to avoid circadian influences. Plasma was stored at -80°C, and BNP concentrations were determined with an immunoradiometric assay (Shionoria, Osaka, Japan). Plasma EPO levels were measured using the IMMULITE® EPO assay (DPC, Los Angeles, CA), which has been described before (11). The DPC assay consists of a ligand-labeled monoclonal anti-EPO capture antibody, an alkaline phosphatase-labeled polyclonal conjugate antibody, and solid-phase anti-ligand-coated polystyrene beads. The amount of plasma EPO was quantified by chemiluminescent measurement in a luminometer. The assay showed an intra-assay variability of less than 1%. The impact of age and gender on plasma EPO levels was limited and not significant.

Renal Function

The glomerular filtration rate (GFR) is a standard indicator of renal function. Under steady-state conditions, GFR is estimated from serum creatinine using a formula that accounts for the influence of age and body weight on creatinine production (the Cockroft Gault equation) GFR:[140-age in years)x(body weight in kg)]/(72xserum creatinine in mg/dL) (12). In women, the value is multiplied by 0.85. This formula is validated and used in several studies of CHF and renal function(13;14).

Statistics

Data are given as mean ± SEM and as frequencies for categorical variables. We included the following risk factors in our analysis: sex, age, history of hypertension, history of diabetes, etiology of the CHF, left ventricular end diastolic dimension (LVEDD), GFR_c, sodium levels, concomitant medication and severity of CHF, assessed by BNP-levels, LVEF and VO_2. Kaplan Meier method was used to study the influence of several baseline clinical and biochemical variables on survival in the study population. The variables with $p < 0.05$ in the univariate analysis were used in the multivariate analyses with the use of the Cox backward Wald regression analysis. Hazard ratios (HR) with 95% confidence intervals (CI) demonstrate the risk of death. Pearson and Spearman correlation coefficients were calculated to determine which clinical and biochemical variable had an univariate correlation with log(EPO). All reported probability values were 2-tailed, and a p-value <0.05 was considered statistically significant. For all statistical analysis SPSS version 11.0 was used.

Table 1. Baseline Characteristics

Variable	Total cohort (n=74)	Survivors (n=52)	Non-Survivors (n=22)	p-value
Age (years)	60.9 ± 1.7	59.3 ± 2.0	64.5±3.4	0.15
Sex (% male)	73.0	78.9	59.1	0.08
NYHA (%)				0.001
Class II	36.5	48.1	9.1	
Class III	32.4	36.5	22.7	
Class IV	31.1	15.4	68.2	
Ischemic etiology (%)	47.3	46.2	50.0	0.76
History of hyptertension (%)	40.6	36.7	50.0	0.31
History of diabetes (%)	12.2	11.5	13.6	0.80
LVEF (%)	0.31± 0.01	0.33 ± 0.02	0.26 ± 0.02	0.035
VO_2 (ml $kg^{-1}min^{-1}$)	19.1 ± 0.6	20.4 ± 0.7	16.1 ± 1.0	0.001
Calculated GFR (ml/min)	76.0 ± 3.5	79.5 ± 4.0	67.0 ± 6.8	0.10
BNP (pmol/L)	109.9 ± 13.5	80.6 ± 12.9	179.1 ± 29.3	0.002
Sodium (mmol/L)	137.6 ± 0.3	137.8 ± 0.4	137.4 ± 0.7	0.58
Erythropoietin levels (mU/mL)	19.4 ± 1.5	16.8 ± 1.3	25.8 ± 3.8	0.033
Hemoglobin levels (g/dL)	13.9 ± 0.2	14.2 ± 0.2	13.3 ± 0.2	0.013
Left Atrial Diameter (mm)	63.6 ± 1.5	62.7 ± 1.7	65.6 ± 2.8	0.36
LV End Systolic Diameter (mm)	49.0 ± 1	47.0 ± 2	53.0 ± 2	0.027
LV End Diastolic Diameter (mm)	60 ± 1	59.0 ± 1	62.0 ± 2	0.28
Medication (% use)				
Ace-inhibitor	81.1	71.2	90.9	0.16
Beta Blocker	67.6	71.2	59.1	0.21
Diuretics	63.5	53.8	86.4	0.009
Aldosteron antagonist	27.0	24.1	39.1	0.24
Nitrates	18.9	13.5	31.8	0.07
Digoxine	27.0	23.1	36.4	0.24

LVEF= left ventricular ejection fraction. VO_2=peak oxygen consumption. LV= left ventricular. ACE= angiotensin covert-ing enzyme. p-value: survivors vs. non-survivors

Results

Patient characteristics

The cohort was 80% male, and age ranged from 26 to 90 years. The NYHA functional class II, III and IV comprised 37%, 32% and 31% respectively. Other baseline characteristics are given in table 1. Of the 74 patients, 22 (30%) died after 16 to 1728 days (mean 621 days), 77.8% of them had a cardiovascular cause of death. Mean follow-up period of the 52 survivors was 1100 days (range 844 – 1934, median 987).

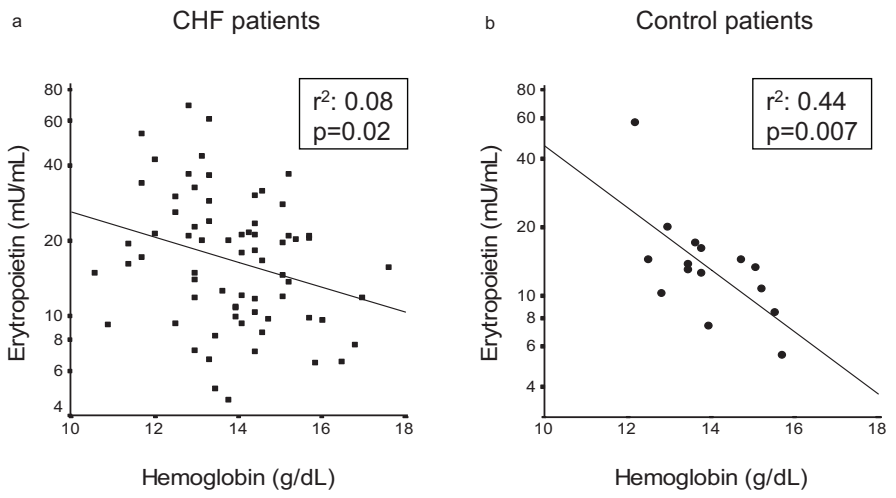


Figure 1a Correlation between log(EPO) and hemoglobin in the CHF cohort. **Figure 1b** Correlation between log(EPO) and hemoglobin in the control patients.

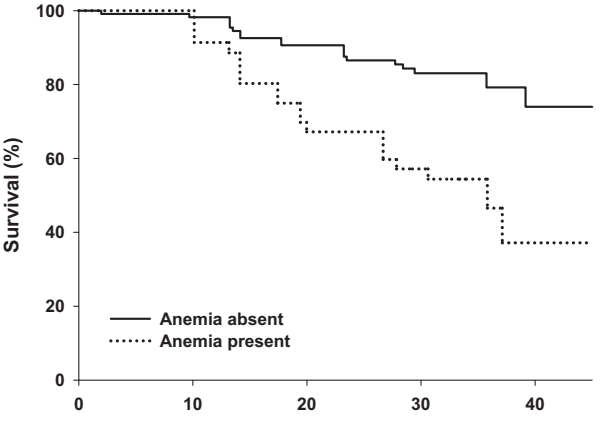


Figure 2 Kaplan-Meier survival curve for CHF patients with and without anemia.

Hb and EPO levels

Mean Hb levels were 13.9 g/dL (range 10.6-17.6 g/dl, median 13.8 g/dL). When we used the generally accepted definition of the World Health Organization (hemoglobin ≤12 g/dL for women, ≤13 g/dl for men), we found that 24.3% of the CHF patients were anemic. Non-survivors had lower Hb levels ($p<0.05$) and higher plasma EPO levels than survivors ($p<0.05$). When log(EPO) was plotted as a function of Hb, only a modest inverse correlation ($r^2=0.08$, $p = 0.02$) was observed in CHF patients (fig 1a). To compare the EPO response in non-CHF patients, we obtained blood samples from 15 control patients. Interestingly, we found a clear, significant inverse correlation between log(EPO) and Hb in the control patients ($r^2=0.44$, $p = 0.007$) (fig 1b).

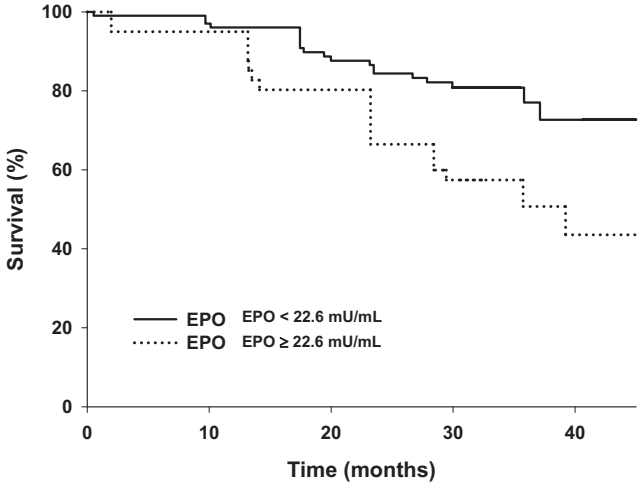


Figure 3 Kaplan-Meier survival curve for the CHF cohort by EPO level

Furthermore, a modest but statistically significant correlation was observed between plasma EPO levels and the severity of CHF, assessed by BNP levels (r^2=0.14, p < 0.001), NYHA-class (r^2=0.09, p=0.01) and VO_2 (r^2=0.09, p=0.01). There was no significant correlation between EPO levels and LVEDD and LVEF, r^2=0.003 and r^2=0.04, respectively. Use of medication, including ACE-inhibitors, betablockers and diuretics, was not significantly correlated to Hb or EPO levels.

Relationship of Hb and EPO with mortality

Kaplan-Meier survival curve clearly demonstrate increased mortality in patients with anemia, HR 3.28 [95% CI; 1.31-8.18, p = 0.01] (fig 2). Patients with anemia had a two year mortality rate of 33 %, compared with 14% for non-anemic patients. Furthermore, also elevated EPO levels (≥ 22.6 mU/mL) are associated with an impaired outcome, HR 2.6 [95% CI; 1.05-6.44, p = 0.04] (fig 3). Two-year mortality rates were 32% in those with high EPO levels (≥22.6 mU/mL), compared with 16% for those with low EPO levels (<22.6 mU/mL). After adjustment for possible confounders, both Hb and EPO levels remained independently associated with an increased mortality (table 2).

Discussion

This is the first study to examine the prognostic value of EPO levels in CHF patients. Our findings demonstrate that increased plasma EPO levels predict an impaired medium to longer-term survival in patients with CHF. Furthermore, as previously described, lower Hb levels were also associated with increased mortality(2). However, EPO and Hb were both independent prognostic markers. When the correlation between EPO and Hb was further studied in CHF patients and compared with controls, we observed an inadequate increase in EPO levels relative to the hemoglobin levels.

We found that 24% of the CHF patients were anemic, according to World Health Organization criteria and Hb levels were an independent predictor of all-cause mortality. These findings are well comparable with others studies, which show that anemia is associated with an

Table 2. Univariate and multivariate predictors of all-cause mortality.

Variable	Univariate			Multivariate		
	HR	CI	p	HR	CI	p
LVEF (%)	0.960	0.920-1.001	0.059	-	-	-
BNP (pmol/L)	1.006	1.003-1.009	0.001	-	-	-
Hb (g/dL)	0.463	0.278-0.773	0.003	0.408	0.219-0.759	0.005
EPO (mU/mL)	1.042	1.015-1.070	0.002	1.034	1.004-1.064	0.026
Age (years)	1.025	0.994-1.056	0.110	1.031	0.999-1.065	0.056
GFRc (ml/min)	0.984	0.965-1.003	0.096	-	-	-

impaired survival in patients with CHF (3;5;15). A recent study of Horwich *et al.* also showed that anemia was common in patients with advanced heart failure (NYHA class III and IV) and patients with NYHA class IV were more likely to have lower Hb levels(2). They also showed that Hb levels <12.3 g/dL were associated with an impaired survival in patients with advanced chronic heart failure. However, the prognostic value of Hb levels in new cases of heart failure seems to be limited(16). None of these studies assessed the role of endogenous EPO levels in CHF patients.

We observed that elevated plasma EPO levels were associated with severity of CHF. This finding has previously been reported by two other groups (8;9). However, we are the first to show that elevated EPO levels are a prognostic marker for impaired survival in the CHF population. Furthermore, elevated plasma EPO was a risk factor independent of Hb levels.

To further elucidate the effect of EPO on Hb levels in CHF patients, we correlated log(EPO) to Hb levels. Interestingly, we found only a very modest inverse correlation between log(EPO) and Hb levels in CHF patients, while there was a clear correlation in the control group. These findings may indicate a blunted EPO response relative to the Hb levels. This mechanism has already been proposed by others, who suggest that resistance to EPO in the bone marrow may explain the anemia observed in CHF patients(17).

From the modest correlation between EPO and Hb in CHF patients, we hypothesize that sensitivity to EPO may be insufficient in CHF patients. Therefore one might assume beneficial effects of EPO treatment in CHF patients(18). Several studies already indicated the positive effects of EPO treatment in CHF patients (4;19;20). A study of Silverberg showed that the treatment of anemia in patients with severe CHF, with EPO and intravenous iron, improved cardiac function and quality of life, and markedly reduced hospitalization(19). Another group observed that EPO treatment in NYHA class III-IV patients, enhanced exercise capacity and also improved quality of life(20). These studies showed that EPO treatment is both safe and beneficial in anemic CHF patients.

The main limitation of this study was its relatively small size (74 patients, 22 events). Furthermore, we did not measure cytokine levels, which may influence the effect of EPO on Hb levels. We measured EPO and Hb levels on a single point and thus can only speculate on its importance over time. Because of these limitations, we regard our study mainly as a hypothesis-generating study. Nevertheless, our findings suggest that lower Hb levels and elevated EPO levels are both independently associated with an impaired survival in CHF patients.

Acknowledgments

We thank dr. H.L. Hillege for expert statistical advice.

References

1. Cromie N, Lee C, Struthers AD. Anaemia in chronic heart failure: what is its frequency in the UK and its underlying causes? Heart 2002;87:377-8.

2. Horwich TB, Fonarow GC, Hamilton MA, MacLellan WR, Borenstein J. Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. J Am Coll Cardiol 2002;39:1780-6.

3. Mozaffarian D, Nye R, Levy WC. Anemia predicts mortality in severe heart failure: the prospective randomized amlodipine survival evaluation (PRAISE). J Am Coll Cardiol 2003;41:1933-9.

4. Silverberg DS, Wexler D, Blum M *et al*. The use of subcutaneous erythropoietin and intravenous iron for the treatment of the anemia of severe, resistant congestive heart failure improves cardiac and renal function and functional cardiac class, and markedly reduces hospitalizations. J Am Coll Cardiol 2000;35:1737-44.

5. Ezekowitz JA, McAlister FA, Armstrong PW. Anemia is common in heart failure and is associated with poor outcomes: insights from a cohort of 12 065 patients with new-onset heart failure. Circulation 2003;107:223-5.

6. Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. 1957. J Am Soc Nephrol 2000;11:589-90.

7. Lorentz A, Eckardt KU, Osswald PM, Duchow JR. Erythropoietin levels in patients depositing autolo gous blood in short intervals. Ann Hematol 1992;64:281-5.

8. Volpe M, Tritto C, Testa U *et al*. Blood levels of erythropoietin in congestive heart failure and correla tion with clinical, hemodynamic, and hormonal profiles. Am J Cardiol 1994;74:468-73.

9. Chatterjee B, Nydegger UE, Mohacsi P. Serum erythropoietin in heart failure patients treated with ACE-inhibitors or AT(1) antagonists. Eur J Heart Fail 2000;2:393-8.

10. Remme WJ, Swedberg K. Guidelines for the diagnosis and treatment of chronic heart failure. Eur Heart J 2001;22:2217-8.

11. Benson EW, Hardy R, Chaffin C, Robinson CA, Konrad RJ. New automated chemiluminescent assay for erythropoietin. J Clin Lab Anal 2000;14:271-3.

12. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron 1976;16:31-41.

13. Hillege HL, Girbes AR, de Kam PJ *et al*. Renal function, neurohormonal activation, and survival in patients with chronic heart failure. Circulation 2000;102:203-10.

14. Robertshaw M, Lai KN, Swaminathan R. Prediction of creatinine clearance from plasma creatinine: comparison of five formulae. Br J Clin Pharmacol 1989;28:275-80.

15. Al-Ahmad A, Rand W.M, Manjunath G *et al*. Reduced kidney function and anemia as risk factors for mortality in patients with left ventricular dysfunction. J Am Coll Cardiol 2003;38:955-62.

16. Kalra PR, Collier T, Cowie MR *et al*. Haemoglobin concentration and prognosis in new cases of heart failure. Lancet 2003;362:211-2.

17. Silverberg DS, Wexler D, Iaina A. The importance of anemia and its correction in the management of severe congestive heart failure. Eur J Heart Fail 2002;4:681-6.

18. Van der Meer P, Voors AA, Lipsic E, van Gilst WH, Van Veldhuisen DJ. Erythropoietin in cardiovas cular diseases. Eur Heart J 2004, 25:285-291

19. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized con trolled study. J Am Coll Cardiol 2001;37:1775-80.

20. Mancini DM, Katz SD, Lang CC, LaManca J, Hudaihed A, Androne AS. Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. Circulation 2003;107: 294- 9.

Part 2: Pleiotropic effects of Erythropoietin

Chapter 5

Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury

Peter van der Meer, Erik Lipsic, Robert H. Henning, Rudolf A. de Boer, Albert J.H. Suurmijer, Dirk J. van Veldhuisen, Wiek H. van Gilst

European Journal of Heart Failure 2004; 6:853-859

Abstract

Background: Recent studies show that erythropoietin (EPO) plays a protective role in brain ischemia. In this condition, administration of EPO protects neurons from ischemic damage. Recently, it has been shown that in patients with chronic heart failure (CHF), EPO treatment improved cardiac function. In the present study we assessed the role of EPO and EPO-receptor (EPO-R) in the heart.

Methods and Results: We studied the presence and functionality of the EPO-R in isolated rat hearts in the Langendorff set-up. Hearts were perfused for 20 minutes with 10 U/ml EPO or vehicle. Immunohistochemistry revealed the presence of the EPO-R on endothelial cells, fibroblasts and to a lesser extent cardiomyocytes. Furthermore, perfusion with EPO resulted in a 50% increase in the phosphorylated MAP kinases p42/p44. To evaluate the protective role of EPO in cardiac ischemia, we performed low-flow (0.6 ml/min) ischemia/reperfusion experiments in isolated rat hearts. Administration of EPO (10 U/ml) reduced the cellular damage by 56% ($p<0.05$) during reperfusion, diminished apoptosis by 15% ($p<0.05$) and resulted in a significantly improved recovery of left ventricular pressure ($p=0.02$) and coronary flow ($p=0.01$).

Conclusion: The present data suggest that a functional EPO-R is present in rat adult cardiac tissue and that exogenous EPO administration improves cardiac function after ischemia/reperfusion injury.

Introduction

In response to ischemia, mammalian cells express a variety of proteins, including Eryhtropoietin (EPO) and Vascular Endothelial Growth Factor (VEGF)(1). The regulation of these two proteins is mediated by hypoxia inducible factor 1 (HIF-1). Expression of HIF-1 increases exponentially, as cellular O_2 concentrations decrease(1;2). Erythropoietin (EPO) is a glycoprotein hormone, primarily produced in the kidney. It mediates the physiological response to hypoxia by increasing red blood cell production. However, expanding evidence suggests that EPO plays also major role in non-erythropoietic processes.

Several reports showed its efficacy in brain and retinal diseases (3-5). A study in rats subjected to cerebral ischemia showed a significant reduction in brain infarct size(5;6). Specificity and biological relevance of these changes were demonstrated by the observation that neutralization of endogenous EPO with soluble EPO-R augments ischemic brain damage(7). During ischemia, the EPO-receptor (EPO-R) is locally upregulated in brain tissue (8). After binding with its receptor, EPO signals through various intracellular pathways, including the MAP p42/p44 and JAK2-STAT5 tyrosine kinases (9). It was recently shown that activation of these pathways by EPO resulted in anti-apoptotic effect in various tissues including, brain, retinal cells and erythroide precursor cells(3;10;11).

Little is known about the presence and protective role of EPO and its receptor in the heart. Juul *et al* have described the presence of EPO and EPO-R in human fetal cardiac tissue (12). Experiments with knock-out mice, deficient for the genes expressing EPO and EPO-R, provide more evidence for its role in cardiac tissue, as both $EPO^{-/-}$ and $EPO\text{-}R^{-/-}$ mice suffer from ventricular hypoplasia and abnormalities in the vascular network (13). Silverberg *et al* have shown that EPO treatment, in patients with CHF, results in an increased left ventricular ejec-

tion fraction, as compared with the placebo control group and there is a growing interest in this subject in the last few years (14;15).
The present study was designed to examine the presence and functionality of the EPO-R in adult cardiac tissue. In addition, we evaluated the protective effects of exogenous EPO administration in ischemia/reperfusion injury in the isolated rat heart.

Methods

Study design

Langendorff experiments were performed in isolated rat hearts that were perfused with EPO (10 U/ml) or vehicle for 20 minutes. These hearts were used to determine the expression and localization of EPO-R, and common signaling pathways were explored. To determine the protective effects of EPO treatment on ischemia/reperfusion injury, we studied 2 experimental groups (each consisting of 6 rats): ischemia/reperfusion without EPO and ischemia/reperfusion with EPO.

Langendorff perfusion:

This well established experimental set-up has been described earlier (11;16-19). In short, rats were anaesthetised with isoflurane in O_2/N_2 and 500U of heparin was injected in the tail vein. The heart was rapidly excised and the aorta was immediately perfused retrogradely by a modified Tyrode solution (glucose 10, NaCl 128.3, KCl 4.7, $NaHCO_3$ 20.2, $CaCl_2$, 1.35, NaH_2PO_4 0.42, $MgCl_2$, 1.05; all mmol/liter) and was equilibrated with 95% O_2 and 5% CO_2. Perfusion pressure was maintained at 60mmHg. Coronary flow (CF) was measured by a microprocessor, which controlled the perfusion pressure by adjusting a peristaltic perfusion pump. CF and left ventricular peak pressure were monitored continuously. After equilibrating for 15 minutes, hearts were subjected to low flow ischemia (0.6ml/min) for 40 minutes, followed by a 2 hours reperfusion period at a constant 60mmHg perfusion pressure. EPO (10U/ml) or vehicle was administered from stabilization throughout the protocol. All the experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutions of Health.

Analysis of coronary effluent

During stabilization (t=5 minutes), ischemia (t=20, t=30 and t=54 minutes) and reperfusion (t=55, t=56, t=57, t=60, t=70, t=90, t=120 and t=150 minutes), coronary perfusate samples were collected. Purines, a sensitive indicator of myocardial ischemia, were determined by high-performance liquid chromatography (HPLC) as previously described(20;21). The total amount of purines released during ischemia and reperfusion, corrected for coronary flow and left ventricular weight, was calculated (area under the curve).

RT-PCR

Snap-frozen LV tissues were used to extract total RNA. Total RNA was isolated using the method of acid guanidium thiocyanate lysis (22). RNA was quantified using a GeneQuant II (Pharmacia Biotechnology). First strand cDNA was synthesized from 1 µg RNA using the RT-PCR Core kit (Perkin-Elmer). Reverse transcriptase (RT) PCR for EPO-R was performed using a forward (5'- AGGACACCTACCTGGTATTGGA-3') and reverse primer (5'-CAGGCCCAGAGA-

GGTTCTCA-3'), yielding a product of 73 bp. To determine the specificity of the PCR reaction the amplicon was digested with Nci I to obtain the expected 39 bp and 34 bp fragments.

Western blotting

Snap frozen LV tissues were homogenized in Radio-Immuno-Precipitation-Assay (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mM -mercaptoethanol, 10 mg/ml PMSF, 5 µl/ml aprotinin, 100 mM sodium orthovanadate, 5 µl/ml benzamidine, 5 µl/ml pepstatine A, 5 µl/ml leupeptine in 1× PBS). Protein concentrations were determined using the DC assay (Bio-Rad) with a bovine albumin standard. Protein levels of EPO-R, phosphorylated MAP kinases p42/p44 and phosphorylated STAT5 were determined by Western blot. Protein samples (50 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes, followed by staining with Ponceau S solution (Sigma). Membranes were incubated with primary antibody against phosphorylated MAP kinases p42/p44 (1:1000 dilution, New England Biolabs), phosphorylated STAT5 (1:1000 dilution, Upstate biotech) and EPO-R (1:250 dilution; C-20 and M-20, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2000, Santa Cruz Biotechnology) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham) and quantified by densitometry.

ImmunIohistochemistry

For immunohistochemistry, cryosections (4 µm) from a midpapillary slice of the left ventricle were fixed in acetone. Hereafter, sections were incubated with two different polyclonal anti-EPO-R antibodies (1:50) (M-20, C-20; Santa Cruz). A two-step indirect peroxidase detection system was employed to visualize the expression pattern of the EPO-R. Sections of placenta and breast carcinoma were used as a positive control(23;24). Slides omitting the primary antibody and preincubation with blocking peptide (10:1) were used as negative controls. For apoptosis detection, sections were incubated with an antibody that specifically recognizes the active form of caspase-3 (1:50; New England Biolabs), as previously reported(25). For quantitative analysis, active caspase-3 positive cells in thirty random fields per section (80-120 cells per field) were counted at high-power magnification. Tissue sections of colonic adenocarcinoma served as a positive control(26;27).

Statistical Analysis

Values shown are mean ± SEM. We used a linear regression model with repeated measures to compare the functional responses to EPO treatment. Data regarding the purine overflow and caspase-3 immunohistochemistry were analyzed by Student's t-test. Statistical significance was defined as $p<0.05$.

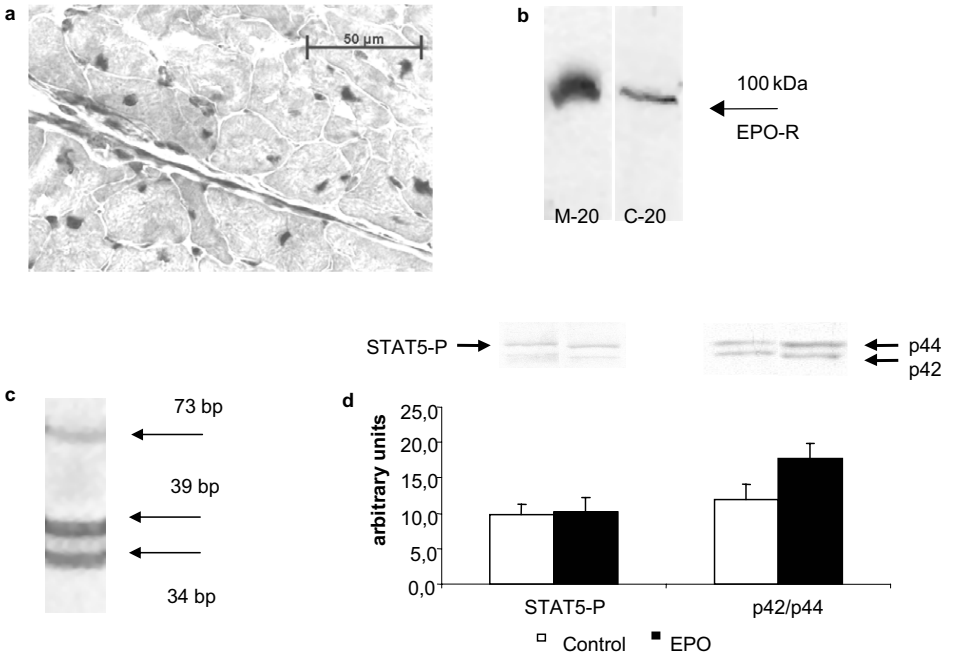


Figure 1a, Immunostaining for EPO-R in normal cardiac tissue. Staining is predominantly observed in interstitial cells, including endothelial cells and fibroblasts. Weak staining is observed in cardiomyocytes.**1b**, Western blot analysis of EPO-R expression in non-ischemic tissue with two different antibodies (M-20, C-20). **1c**, RT-PCR analysis of EPO-R mRNA transcripts (73 bp), after partial digestion with restriction enzyme Nci I, two specific products (39 bp and 34 bp) are obtained. **1d**, Effects of EPO (10 U/ml) on phosphorylated STAT5 (92 kDa) and MAP kinase p42/p44 (42 and 44 kDa), assessed by Western blot analysis (n=6).

Results

Expression pattern and functionality of EPO-R in heart

We determined the expression of EPO-R in normal cardiac tissue by immunohistochemistry. Immunostaining for EPO-R was predominantly observed in interstitial cells, including endothelial cells and fibroblasts. Cardiomyocytes showed weak expression of the EPO-R (Fig 1a). We found similar expression patterns with both antibodies, while incubation with 10x excess of blocking peptide completely abolished the signal (data not shown). Western blotting revealed a specific signal for the EPO-R with both antibodies at the expected size of 100 kDa (Fig 1b). Further, we studied the expression levels of the EPO-R after ischemia-reperfusion injury. In the group without EPO perfusion, we did not observe a change in the expression level of the EPO-R compared to non-ischemic tissue. However, EPO treatment during ischemia-reperfusion induced a 26 ± 8.3% downregulation of the EPO-R. Further, RT-PCR revealed EPO-R gene transcription in the rat heart. Specificity of the product was confirmed by restriction fragment length analysis (Fig 1c). We further explored potential signal transduction pathways of the EPO-R, by infusing 10 U/ml EPO in Langendorff-perfused hearts for twenty minutes. This resulted in a 50% increase in levels of phosphorylated MAP kinase p42/44 in the left ventricles of EPO-perfused hearts compared with vehicle perfused hearts (Fig 1d). No increase in the levels of phosphorylated STAT5 were detected.

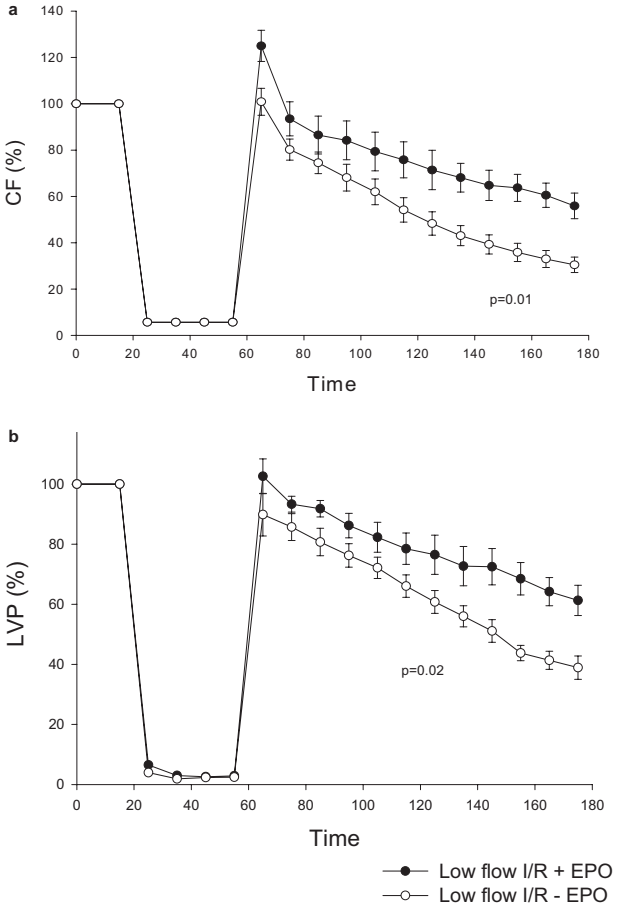


Figure 2. Effects of EPO treatment on LVP (2A) and CF (2B). Values are shown as a percentage from baseline (n=12).

Effects of EPO on Cardiac Function

To test the potential protective effects of EPO in the heart, we performed low-flow ischemia/reperfusion experiments in isolated rat hearts. Baseline characteristics, body weight, heart weight, CF and LVP did not differ between both groups (data not shown). During low-flow ischemia the cardiac function decreased to a similar extent in the two groups, irrespective of EPO treatment. During reperfusion, post-ischemic hyperemia occurred in both groups, but CF was restored to a significantly higher level during the two hours reperfusion period in the EPO treated group ($p=0.02$, Fig 2a). Furthermore, LVP was significantly increased throughout the reperfusion period in the EPO treated group compared with the vehicle treated group ($p=0.01$, Fig 2b).

Effects of EPO on Cellular Damage

To determine the effect of EPO perfusion on cellular damage, we measured purine overflow at different time points during stabilization, ischemia and reperfusion period. Total overflow of purines during reperfusion showed a 56% decrease (711 ± 183 nmol/g versus 1614 ±

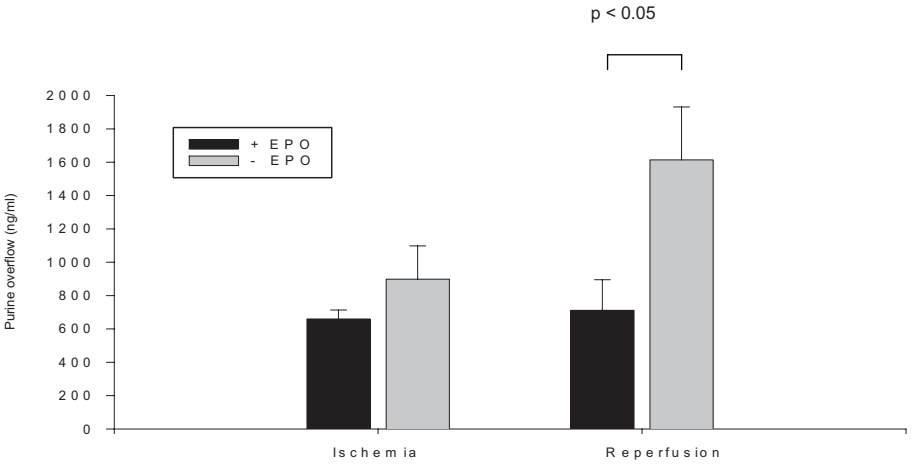


Figure 3. Effect of EPO perfusion on total purine overflow during ischemia and reperfusion (area under the curve). Purines are a marker for ATP breakdown and therefore an indicator of reversible and irreversible damage to the myocardium (n=12).

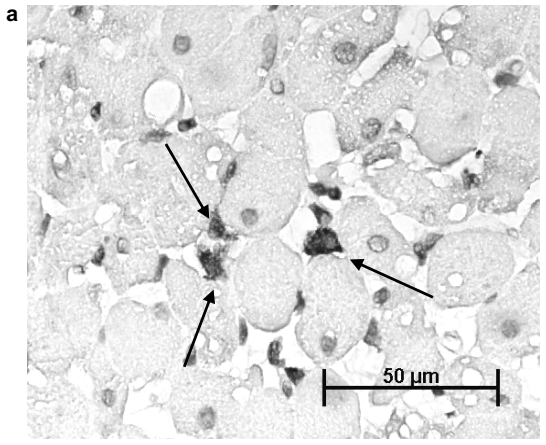
317 nmol/g) ($p<0.05$) in the EPO treated group, compared with the vehicle treated hearts. A smaller difference was observed during the ischemic period between the EPO perfused hearts and the control group, 660 ± 53 nmol/g versus 898 ± 200 nmol/g, respectively (p=NS; Fig 3). No purines were detected at baseline.

Furthermore, we studied the anti-apoptotic effects of EPO perfusion on the heart. Staining with anti-active caspase-3 was mostly restricted to endothelial cells and fibroblasts (Fig 4a). The hearts perfused with EPO demonstrated a 15% reduction in apoptotic cells (2.1% ± 0.12 versus 1.8% ± 0.09) ($p<0.05$, Fig 4b).

Discussion

In the present study, we demonstrated the presence of a functional EPO-R in adult cardiac tissue and we showed that EPO administration limited cardiac damage and preserved cardiac function after ischemia/reperfusion injury. However, the mechanism by which EPO preserves cardiac function is currently unknown.

We found that EPO stimulation increases the levels of phosphorylated MAP kinases p42/p44 in normal rat heart. This pathway has already been implicated as a survival pathway in cardiac cells after ischemia/reperfusion injury, by inhibiting apoptosis (28-30). A study by Yue *et al.* provided more evidence for the role of MAP kinases in ischemia-reperfusion injury by demonstrating that inhibition of the MAP kinases p42/p44 pathway exacerbated cardiac injury and showed a diminished functional recovery compared with control hearts(31). Thus, activation of this pathway seems to be important for survival of cardiac cells by protecting them from programmed cell death. With respect to STAT5, we did not observe difference in the amount of phosphorylated STAT5 after EPO perfusion for twenty minutes. This might be related to different time-points of STAT5 phosphorylation after perfusion with EPO. Furthermore, this pathway could play a minor role in the cardiac EPO signaling, as shown in vascular



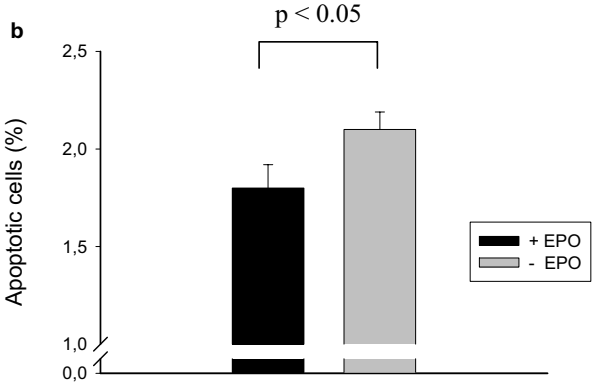


Figure 4. Immunostaining for active caspase-3 in ischemic cardiac tissue without EPO treatment. 4B. Percentage of active caspase-3 positive cells at the end of reperfusion in isolated rat hearts perfused with or without EPO and subjected to 40 min. of low-flow ischemia and 2 hours of reperfusion (n=12).

smooth muscle cells by Ammarguellat *et al* (11;32). Future experiments will be needed to precisely explore the EPO signaling pathways in the heart.

Both ischemic and reperfused myocardium can undergo apoptosis, however, during reperfusion, accelerated apoptosis occurs in cardiac cells(33). We observed that EPO limits cardiac damage by 56% during reperfusion. A recent paper from Scarabelli *et al* showed that in the early stages of reperfusion, apoptosis is first seen in endothelial cells and is spreading to surrounding cardiac myocytes, suggesting that reperfusion induces the release of pro-apoptotic mediators from endothelial cells(34). We found that the EPO-R was predominantly localized to endothelial cells and fibroblasts. Interestingly, we observed in these cells a reduction of apoptosis of 15%, when the hearts were perfused with EPO. By preventing apoptosis in these cells, it is tempting to speculate that EPO can preserve vascular flow and ultimately protect the myocardium. Although a reduction of 15% in apoptotic cells seems modest, recent in-

vestigations suggests that apoptosis after myocardial infarction is progressive, and therefore small amounts of apoptotic cells may result in more extensive cell loss(35). Recent data reported on the beneficial effects of preconditioning in the rodent heart in which exposure of wild-type mice to intermittent hypoxia resulted in protection from ischemia-reperfusion injury(36). Ischemic preconditioning was absent in mice heterozygous for a knockout in the HIF-1α gene. Further, in wild-type mice, EPO administration at 24 hours prior to *ex vivo* ischemia-reperfusion resulted in a reduction in apoptosis and an increased cardiac recovery. While these findings are in accordance with our results the present study suggests that there is no need for an extended period of pretreatment for EPO to exert its protective effects.

In addition to its anti-apoptotic effects, EPO may protect the myocardium through other mechanisms that have not been assessed in this work. Oxidative stress plays an import role in the reperfusion damage observed in the myocardium(37). Recent research suggests that EPO can also directly protect tissue against the effects of free radicals(38). Furthermore, it has been shown that EPO may increase the Nitric Oxide (NO) production when EPO-induced erythrocytosis occurs, reviewed by Smith *et al.*(39). Transgenic mice overexpressing human erythropoietin showed higher NO synthase levels and an increased NO-mediated endothelium derived relaxation(40). On the other hand, Noguchi *et al* showed that one-week of erythropoietin treatment in rabbits, resulted in a decreased response to endothelium dependent vasodilators(41). EPO has also been shown to act as a cardioprotective agent, by modulating the cardiac Na^+-K^+-pump(42).

EPO has been widely used in clinical practice for more than a decade. A recent study of Silverberg *et al*. showed the beneficial effects of rh-EPO therapy in CHF patients(14). They conducted a placebo controlled study in 32 mild anemic patients with severe CHF (NYHA $\geq$III) and treated them with rh-EPO. Over a mean of 8.2 +/- 2.6 months, left ventricular ejection fraction increased 5.5% in the treatment group, compared to a decrease of 5.4% in the control group. These results strongly suggest an important role for rh-EPO in patients with CHF. Although correction of anemia has beneficial effects on cardiac function, non-erythropoietic effects are also likely to play a role. More evidence for non-erythropoietic effects of EPO in human was provided by Ehrenreich *et al* (43). They recently conducted a pilot double blind randomized clinical trial to investigate the acute effects of EPO treatment in patients with ischemic stroke. Administration of EPO within 8 hrs after stroke reduced brain infarct size and improved the clinical outcome. As there are many similarities between brain and heart ischemia, EPO administration may become an adjunctive therapy for the treatment of acute coronary syndromes. Further work is needed to determine the mechanisms by which EPO reduces cardiac damage and preserves cardiac function.

In conclusion, this study suggests that EPO treatment is effective in reducing myocardial damage and preserving cardiac function after ischemia/reperfusion injury. This implies an organ protective role of EPO beyond erythropoiesis and warrants the search for organ specific EPO analogues.

Acknowledgments

The authors thank Egbert Scholtens, Cécile Driessen, Kristien Boddeus and Jacko Duker for expert technical assistance. Peter van der Meer is supported by NWO ZonMW. Erik Lipsic is supported by GUIDE.

References

1. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may medi ate hypoxia-initiated angiogenesis. Nature 1992;359:843-5.

2. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS het erodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-4.

3. Grimm C, Wenzel A, Groszer M *et al*. HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nat Med 2002;8:718-24.

4. Chong ZZ, Kang JQ, Maiese K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. Circulation 2002;106:2973-9.

5. Siren AL, Fratelli M, Brines M *et al*. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A 2001;98:4044-9.

6. Brines ML, Ghezzi P, Keenan S *et al*. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 2000;97:10526-31.

7. Sakanaka M, Wen TC, Matsuda S *et al*. *In vivo* evidence that erythropoietin protects neurons from isch emic damage. Proc Natl Acad Sci U S A 1998;95:4635-40.

8. Siren AL, Knerlich F, Poser W, Gleiter CH, Bruck W, Ehrenreich H. Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. Acta Neuropathol (Berl) 2001;101:271-6.

9. Haq R, Halupa A, Beattie BK, Mason JM, Zanke BW, Barber DL. Regulation of erythropoietin-induced STAT serine phosphorylation by distinct mitogen-activated protein kinases. J Biol Chem 2002;277:17359-66.

10. Chong ZZ, Kang JQ, Maiese K. Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades. Journal of Cerebral Blood Flow and Metabolism 2002;22:503-14.

11. Ratajczak J, Majka M, Kijowski J *et al*. Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. Br. J. Haematol 2001; 115:195-204

12. Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. Early Hum Dev 1998;52:235-49.

13. Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 1999;126:3597-605.

14. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized controlled study. J Am Coll Cardiol 2001;37:1775-80.

15. Silverberg DS, Wexler D, Iaina A. The importance of anemia and its correction in the management of severe congestive heart failure. Eur J Heart Fail 2002;4:681-6.

16. Sato M, Engelman RM, Otani H *et al*. Myocardial protection by preconditioning of heart with losartan, an angiotensin II type 1-receptor blocker: implication of bradykinin- dependent and bradykinin-inde pendent mechanisms. Circulation 2000;102:III346-III351.

17. Pinto YM, Bader M, Pesquero JB *et al*. Increased kallikrein expression protects against cardiac ischemia. FASEB J 2000;14:1861-3.

18. Nawata T, Takahashi N, Ooie T, Kaneda K, Saikawa T, Sakata T. Cardioprotection by streptozotocin-in duced diabetes and insulin against ischemia/reperfusion injury in rats. J Cardiovasc Pharmacol 2002;40:491-500.

19. De Jonge R, Out M, Maas WJ, De Jong JW. Preconditioning of rat hearts by adenosine A(1) or A(3) receptor activation. Eur J Pharmacol 2002;441:165-72.

20. Backstrom T, Goiny M, Lockowandt U, Liska J, Franco-Cereceda A. Cardiac outflow of amino acids and purines during myocardial ischemia and reperfusion. J Appl Physiol 2003;94:1122-8.

21. van Gilst WH, De Graeff PA, Kingma JH, Wesseling H, De Langen CD. Captopril reduces pu rine loss and reperfusion arrhythmias in the rat heart after coronary artery occlusion. Eur J Pharmacol 1984;100:113-7.

22. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phe nol-chloroform extraction. Anal Biochem 1987;162:156-9.

23. Conrad KP, Benyo DF, Westerhausen-Larsen A, Miles TM. Expression of erythropoietin by the human placenta. FASEB J 1996;10:760-8.

24. Acs G, Zhang PJ, Rebbeck TR, Acs P, Verma A. Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. Cancer 2002;95:969-81.

25. De Boer RA, van Veldhuisen DJ, van der WJ *et al*. Additional use of immunostaining for active caspase 3 and cleaved actin and PARP fragments to detect apoptosis in patients with chronic heart failure. J Card Fail 2000;6:330-7.

26. Jonges LE, Nagelkerke JF, Ensink NG *et al*. Caspase-3 activity as a prognostic factor in colorectal carcinoma. Lab Invest 2001;81:681-8.

27. Yang L, Cao ZH, Yan H, Wood WC. Coexistence of high levels of apoptotic signaling and inhibi tor of apoptosis proteins in human tumor cells: Implication for cancer specific therapy. Cancer Re search 2003;63:6815-24.

28. Schulman D, Latchman DS, Yellon DM. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 MAPK signaling pathway. Am J Physiol Heart Circ Physiol 2002;283: H1481-H1488.

29. Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JH, Chien KR. Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. J Biol Chem 1997;272:5783-91.

30. Zu YL, Ai Y, Gilchrist A *et al*. High expression and activation of MAP kinase-activated protein kinase 2 in cardiac muscle cells. J Mol Cell Cardiol 1997;29:2159-68.

31. Yue TL, Wang C, Gu JL *et al*. Inhibition of extracellular signal-regulated kinase enhances Ischemia/Re oxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res 2000;86:692-9.

32. Ammarguellat F, Llovera M, Kelly PA, Goffin V. Low doses of EPO activate MAP kinases but not JAK2-STAT5 in rat vascular smooth muscle cells. Biochem Biophys Res Communtions 2001; 284:1031-8.

33. Fliss H, Gattinger D. Apoptosis in ischemic and reperfused rat myocardium. Circ Res 1996;79:949-56.

34. Scarabelli T, Stephanou A, Rayment N *et al*. Apoptosis of endothelial cells precedes myocyte cell apop tosis in ischemia/reperfusion injury. Circulation 2001;104:253-6.

35. Sam F, Sawyer DB, Chang DL *et al*. Progressive left ventricular remodeling and apoptosis late after myocardial infarction in mouse heart. Am J Physiol Heart Circ Physiol 2000;279:H422-H428.

36. Cai Z, Manalo DJ, Wei G *et al*. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation 2003;107:107-13.

37. Dhalla NS, Elmoselhi AB, Hata T, Makino N. Status of myocardial antioxidants in ischemia-reperfusion injury. Cardiovasc Res 2000;47:446-56.

38. Chattopadhyay A, Choudhury TD, Bandyopadhyay D, Datta AG. Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. Biochem Pharmacol 2000;59:419-25.

39. Smith KJ, Bleyer AJ, Little WC, Sane DC. The cardiovascular effects of erythropoietin. Cardiovasc Res 2003;59:538-48.

40. Ruschitzka FT, Wenger RH, Stallmach T *et al*. Nitric oxide prevents cardiovascular disease and de termines survival in polyglobulic mice overexpressing erythropoietin. Proc Natl Acad Sc USA 2000;97:11609-13.

41. Noguchi K, Yamashiro S, Matsuzaki T *et al*. Effect of 1-week treatment with erythropoietin on the vascular endothelial function in anaesthetized rabbits. British J Pharmacol 2001;133: 395-405.

42. Sterin-Borda L, Barcelo AC, Bozzini CE. Erythropoietin improves cardiac contractility in post-hypoxic mice. Br J Haematol 2003;121:180-6.

43. Ehrenreich H, Hasselblatt M, Dembowski C *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2002;8:495-505.

Chapter 6

Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction

Peter van der Meer, Erik Lipsic, Robert H. Henning, Kristien Boddeus, Jolanda van der Velden, Adriaan A. Voors, Dirk J. van Veldhuisen, Wiek H. van Gilst, Regien G. Schoemaker

Journal of the American College of Cardiology 2005; 46: 125-133

Abstract

Objectives: We assessed the effects of erythropoietin (EPO) treatment in a rat model of post-MI heart failure.

Background: EPO, traditionally known as a hematopoietic hormone, has been linked to neovascularization. Whereas administration of EPO acutely after myocardial infarction (MI) reduces infarct size and improves cardiac function, its role in the failing heart is unknown.

Methods: Rats underwent coronary ligation or sham surgery. Rats with MI were randomly assigned to: untreated (MI), a single bolus of EPO immediately after MI induction (MI-EPO-early), EPO treatment immediately after MI and once every three weeks (MI-EPO-early+late) and EPO treatment starting three weeks after induction of MI, once every three weeks (MI-EPO-late). After nine weeks, hemodynamics, infarct size, myosin heavy chain (MHC) isoforms, myocyte hypertrophy and capillary density were measured.

Results: EPO treatment started immediately after MI (MI-EPO-early and MI-EPO-early+late) resulted in a 23-30% reduction in infarct size ($p<0.01$), and accordingly hemodynamic improvement. EPO treatment, started three weeks after MI (MI-EPO-late), did not affect infarct size, but resulted in an improved cardiac performance, reflected by a 34% reduction in left ventricular end-diastolic pressure ($p<0.01$), and 46% decrease in ANP levels ($p<0.05$). The improved cardiac function was accompanied by an increased capillary density ($p<0.01$), an increased capillary-to-myocyte ratio ($p<0.05$) and a partial reversal of β-MHC ($p<0.05$) in all treated groups.

Conclusion: In addition to its effect on infarct size reduction, EPO treatment improves cardiac function in a rat model of post-MI heart failure. This observation may be explained by neovascularization, associated with an increased α-MHC expression.

Introduction

Erythropoietin (EPO) is best known as a hematopoietic growth factor, promoting proliferation and differentiation of erythroid progenitor cells. However, the expression of the EPO-receptor outside the hematopoietic system, including endothelial cells, cardiomyocytes and neurons, may suggest additional effects of EPO beyond hematopoiesis(1-4).

Since an insufficient amount of capillaries may lead to left ventricular dilatation and heart failure after myocardial infarction (MI) (5), treatment directed towards increasing capillary density might be beneficial in heart failure. Expanding evidence shows that EPO is involved in angiogenesis. It has been shown that stimulation of cultured endothelial cells with EPO resulted in cell proliferation, chemotaxis and differentiation into vascular structures(6). Furthermore, Jaquet *et al* found that EPO and Vascular Endothelial Growth Factor (VEGF) were equally effective in stimulating angiogenesis in endothelial cells derived from the myocardium(7). Most recently, it has been shown that EPO treatment in a rat stroke model, resulted in an increased capillary density around the ischemic lesion(8). In addition, EPO has been implicated to play a protective role during acute ischemia in brain (2;9;10) and heart(11-13).

Pretreatment with exogenous EPO rescued hypoxic cultured cardiomyocytes from apoptosis(12). EPO perfusion during ex-vivo ischemia-reperfusion, improved left ventricular function and reduced cellular damage(4;13;14). Acute, systemic treatment with EPO, in a rodent ischemia-reperfusion model, substantially reduced infarct size and decreased myocardial

apoptosis(12), even when EPO was administered after reperfusion(11;15).
While the cardioprotective effects of EPO during acute MI are increasingly recognized, the role of EPO treatment in chronic heart failure (CHF) is unknown. Therefore, we assessed the effects of EPO treatment in a rat model of post-MI heart failure(16). In this model induction of MI leads to a time and infarct size related ventricular dilatation and heart failure(17). We hypothesized that EPO treatment initiated after heart failure development (three weeks after induction of MI) would improve cardiac performance, possibly by increasing capillary density. To distinguish the acute effects of EPO (i.e. infarct size reduction) from its effects in CHF, we studied two additional groups. In one group we administered only a single dose of EPO immediately after MI, and in a second group we administered EPO immediately after MI and continued EPO treatment during the experiment.

Methods

Animals

We used male Sprague Dawley rats weighing 270-330 g (Harlan, Zeist, The Netherlands). Animals were fed ad libitum, and housed in groups of four to five rats, according to institutional rules with 12:12 hours light-dark cycles. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Design of the study

Rats were either subjected to left coronary artery ligation (n=85) or sham surgery (n=8). Rats with MI were randomized to one of four groups; untreated (MI) or three different treatment strategies with EPO: a single bolus of EPO immediately after ligation (MI-EPO-early), EPO treatment directly after ligation and once every three weeks (MI-EPO-early+late) and EPO treatment starting three weeks after ligation, once every three weeks (MI-EPO-late). EPO (Darbopoetin-alpha; Aranesp) was administered intraperitoneally at a dose of 40 μg/kg, which equals 8.000 U/kg recombinant human EPO (Amgen Inc., Thousands Oaks, CA, USA) and is in close range of known dosages for organ protection(11;12;18). Hematocrit was measured at baseline and at week one, three, four, six and nine after surgery. Persons blinded to the treatment groups performed the analysis of samples obtained from the experiments.

Myocardial infarction model

This model has been described previously(16). Briefly, rats were anesthetized with 2% isoflurane in 2.5L oxygen/minute. After intubation, the rats were put on a mechanical ventilator (frequency 90/min) and a left-side thoracotomy was performed. MI was induced by ligating the proximal portion of the left coronary artery, beneath the left atrial appendage. In sham operated rats, the same surgery was performed, without ligating the suture.

Hemodynamic measurements

After nine weeks rats were anaesthetized as described above. Microtip pressure transducer (Millar Instr. Inc.) was inserted into the left ventricular cavity via the right carotid artery. After a 3-min period of stabilization, heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and developed left ventricular pressure (dLVP=LVSP-LVEDP) were measured. As indices of contractility and relaxation, the maximal

Table 1. Characteristics of the experimental groups

	Sham	MI	MI-EPO-early	MI-EPO-late	MI-EPO-early+late
General					
n	8	12	12	13	13
Infarct size (% of LV)	...	43±3	30±2§	41±3	33±2§
Hemodynamics					
Heart rate (bpm)	313±8	324±6	332±7	326±7	328±8
SBP (mmHg)	127±3	111±4†	115±3†	120±3‡	122±3‡
DBP (mmHg)	78±2	78±2	79±2	83±3	86±2
Body/organ weight					
BW (g)	390±10	395±11	401±8	400±7	421±6
Lungweight/BW (mg/g)	3.9±0.1	6.4±1.0†	4.2±0.5§	3.9±0.1§	3.9±0.2§
Heartweight/BW (mg/g)	3.2±0.1	4.0±0.2†	3.8±0.1†	3.7±0.1*	3.7±0.1*
Hematocrit					
baseline (%)	48±0.6	47±0.3	48±0.5	48±0.6	47±0.6
1 week (%)	48±0.6	47±1.1	58±0.9†,§	46±0.7	59±0.7†,§
3 weeks (%)	50±1.1	49±0.7	53±0.5†,§	49±0.7	53±0.7†,§
4 weeks (%)	50±0.5	51±0.8	50±0.4	62±0.5†,§	64±1.8†,§
6 weeks (%)	50±0.4	50±0.7	49±0.6	60±0.7†,§	61±0.7†,§
9 weeks (%)	44±0.5	44±1.5	44±0.7	54±1.3†,§	56±1.7†,§

Data are presented as mean±SEM. n indicates number of animals; LV, Left Ventricle; SBP, systolic blood pressure; DBP, diastolic blood pressure; BW, bodyweight. *p<0.05; †p<0.01 vs Sham; ‡p<0.05, §p<0.01 vs. MI.

rates of increase and decrease in LVP ($+dP/dt_{max}$ and $-dP/dt_{max}$) were determined. The catheter was retracted into the aortic arch and arterial systolic and diastolic blood pressures (SBP, DBP) were recorded.

Plasma N-terminal ANP levels

Arterial blood was collected after nine weeks, anti-coagulated with EDTA, and plasma was stored at −80˚C until assayed. Plasma N-terminal atrial natriuretic peptide (N-ANP) was measured by a commercially available radioimmuno-assay (Biotop, Oulu, Finland), as described previously(19).

Infarct size and left ventricular hypertrophy

After hemodynamic measurements, hearts were rapidly excised and weighed. Mid-papillary slices were prepared for immunohistochemistry. Slices were fixed in 4% paraformaldehyde and paraffin-embedded. Infarct size was determined by planimetry at mid-ventricular levels in transverse slices on picrosirius red/fast green–stained sections. Infarct size was expressed as the percentage of scar length to total left ventricular circumference, as described previously(20;21). Deparaffinized 5-μm thick sections were stained with a Gomori's silver staining, in order to visualize individual myocytes in the viable LV wall, the area with the most pronounced underperfusion(22). Using image analysis (Zeiss KS 400, Germany), concentric myocyte hypertrophy in the viable LV wall, remote from the infarcted area, was measured as

the cross-sectional area of transversally cut myocytes showing a nucleus. Myocyte density was calculated as the average number of myocytes per tissue area. In each stained section, measurements were averaged from three different counting fields (± 75 myocytes per heart)

Myosin Heavy Chain (MHC) Isoform Analysis

Samples of the left ventricle (not infarct area), were frozen in liquid nitrogen and stored at −80˚C. The freeze dried samples were dissolved in a buffer and gel electrophoresis was performed as described previously(23). Samples (0.5μg) were run at constant current (24 mA) for 5 hours. Silver staining of the gels and laser scanning densitometry was performed to identify differences in myosin isoform composition (i.e.α-MHC and β-MHC).

Capillary density

To visualize capillaries in the myocardium in the same area as used for the measurements of the myocyte size, endothelial cells were stained with biotin-labeled Lectin GSL (1:100; Sigma-Aldrich), as previously described (16). Since Lectins stain not only capillaries but also other vessels, a size criterion of 10 μm was used to exclude small arterioles and venules. Image analysis (Image Pro-plus version 4.5) was used to measure capillary density; calculated as the number of capillaries per tissue area. The measured total tissue area was corrected for the remaining interstitial space. Actual neovascularization was derived from an increased capillary to myocyte ratio, which has been calculated as capillary density divided by myocyte density(24).

Statistical analysis

Data are presented as mean±SEM. Statistical analysis between groups was performed by 1-way ANOVA. When a statistically significant difference was detected, a Fisher's protected LSD post-hoc analysis was performed. Correlation analysis was performed with Pearson's correlation tests. Differences were considered significant at $p<0.05$.

Results

General

Overall mortality following MI was 41%. Mortality occurred only in the first 24 hours after induction of MI. There were no statistically significant differences in mortality between the four groups (MI:50%, MI-EPO-early:40%, MI-EPO-late 32% and MI-EPO-early+late:41%; $p=0.54$). No mortality was observed in sham-operated rats. At baseline, no differences in body weight were observed (data not shown). General characteristics after nine weeks are shown in table 1. Body weight was comparable among the five groups. Among groups with MI, systolic blood pressure (SBP) was significantly lower only in MI and MI-EPO-early compared to sham; $p<0.01$. SBP was significantly higher in MI-EPO-late and MI-EPO-early+late, compared to MI group ($p<0.05$). No significant differences were observed in heart rate and diastolic blood pressure (DBP), although there was a trend towards higher DBP in the groups repeatedly treated with EPO (MI-EPO-late and MI-EPO-early+late). The changes of the hematocrit throughout the experiment are also shown in table 1. After nine weeks hematocrit values were significantly elevated in the MI-EPO-late and MI-EPO-early+late, compared to other groups.

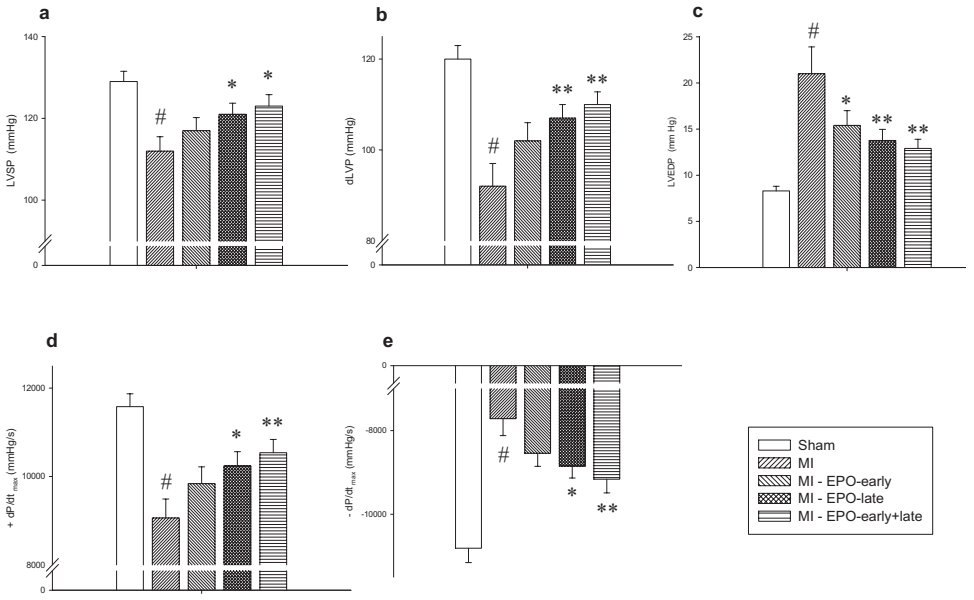


Figure 1. Effects of myocardial infarction and EPO treatment on hemodynamic parameters. LVSP indicates left ventricular systolic pressure; dLVP, developed left ventricular pressure; LVEDP, left ventricular end diastolic pressure; $+dP/dt_{max}$ and $-dP/dt_{max}$, maximal rate of increase and decrease of ventricular pressure, respectively. * $p<0.05$ vs. MI, ** $p<0.01$ vs. MI, # $p<0.01$ vs. Sham

Infarct size

LV-infarct size (% of LV) was comparable between MI and MI-EPO-late, 43% and 41% respectively ($p=0.60$; table 1). Treatment with EPO immediately after coronary artery ligation, reduced infarct size by 30% in MI-EPO-early and by 23% in MI-EPO-early+late group (both $p<0.01$ vs. MI; table 1).

Hemodynamic measurements

Hemodynamic data obtained nine weeks after surgery are summarized in figure 1. LVSP and developed LVP (dLVP) were both clearly diminished in MI compared to sham operated rats ($p<0.01$ for both). MI-EPO-late and MI-EPO-early+late showed a significantly higher LVSP and dLVP, compared to MI (both $p<0.05$). One single bolus of EPO immediately after ligation (MI-EPO-early) did not result in a significantly improved LVSP or dLVP (figure 1A and 1B).
LVEDP was elevated in MI compared to sham operated rats (21 ± 3mmHg vs. 8 ± 1mmHg; $p<0.01$). Importantly, EPO treatment started three weeks after MI (MI-EPO-late), resulted in a 34% decrease in LVEDP, compared to MI ($p<0.01$), despite similar infarct sizes. Immediate treatment with EPO after induction of MI (MI-EPO-early and MI-EPO-early+late) led to a 27% and 38% reduction in LVEDP respectively, compared to MI group ($p<0.05$ and $p<0.01$; figure 1C).
Myocardial contractility ($+dP/dt_{max}$) and myocardial relaxation ($-dP/dt_{max}$) were both impaired in MI compared to the sham group (both $p<0.01$). MI-EPO-late and MI-EPO-early+late showed an improved contractility and relaxation compared to MI (all $p<0.05$). In contrast, when only one single bolus of EPO was administered immediately after MI (MI-EPO-early), contractility

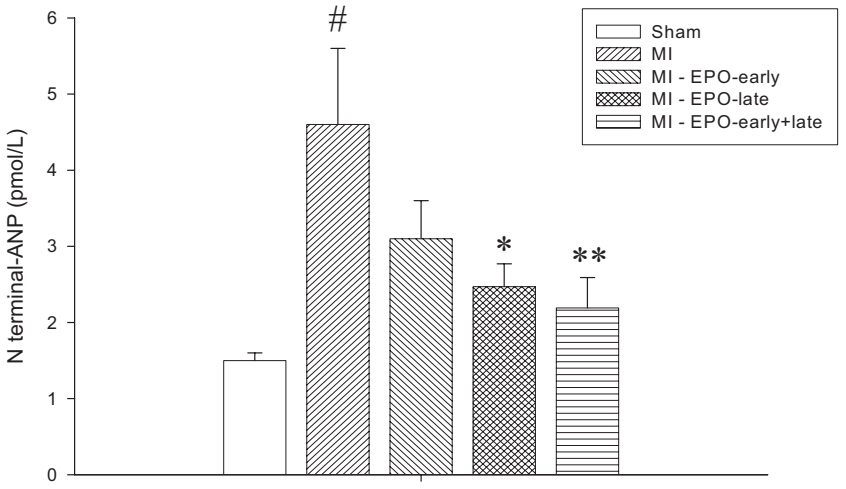


Figure 2. Plasma N-terminal ANP levels. * $p<0.05$ vs. MI, ** $p<0.01$ vs. MI, # $p<0.01$ vs. Sham.

and relaxation were not significantly improved compared to MI (figure 1D and 1E).

N-terminal ANP levels

Figure 2 shows that plasma N-ANP levels were three-fold increased in MI group ($p<0.01$ vs. sham-operated animals). Furthermore, N-ANP levels were significantly reduced in the MI-EPO-late and MI-EPO-early+late groups (both $p<0.05$ vs. MI), returning to sham values (both p=NS vs. sham). The MI-EPO-early group showed a trend towards lower N-ANP levels ($p=0.07$ vs. MI).

Organ weights and LV hypertrophy

As shown in table 1, the ratios of heart weight (HW) to body weight and that of lung weight to body weight were significantly increased in the MI compared to the sham-operated group (both $p<0.01$). Lung weight to body weight (an indirect expression of the LV-end diastolic pressure and thus severity of heart failure) was significantly reduced in all EPO treatment groups (all $p<0.01$ vs. MI). A trend towards lower HW to body weight compared to MI was observed in MI-EPO-late and MI-EPO-early+late groups. LV hypertrophy was further studied by histological analysis. Representative photomicrographs of Gomori stained stections of the viable LV free wall are shown in figure 3A. MI resulted in a 35% increase in myocyte cross-sectional area, compared to sham ($p<0.05$). All EPO treated groups showed a trend towards a smaller myocyte cross-sectional area, although this did not reach statistical significance (figure 3B).

Differences in MHC isoform composition

Relative proportion of cardiac α-MHC and β-MHC were compared in LV protein samples between the five different groups. MI resulted in a more than 5-fold increase in expression of β-MHC, compared to sham operated rats ($p<0.01$). EPO treatment in all three groups reduced the expression of β-MHC by 26-31%, compared to MI ($p<0.05$, figure 4).

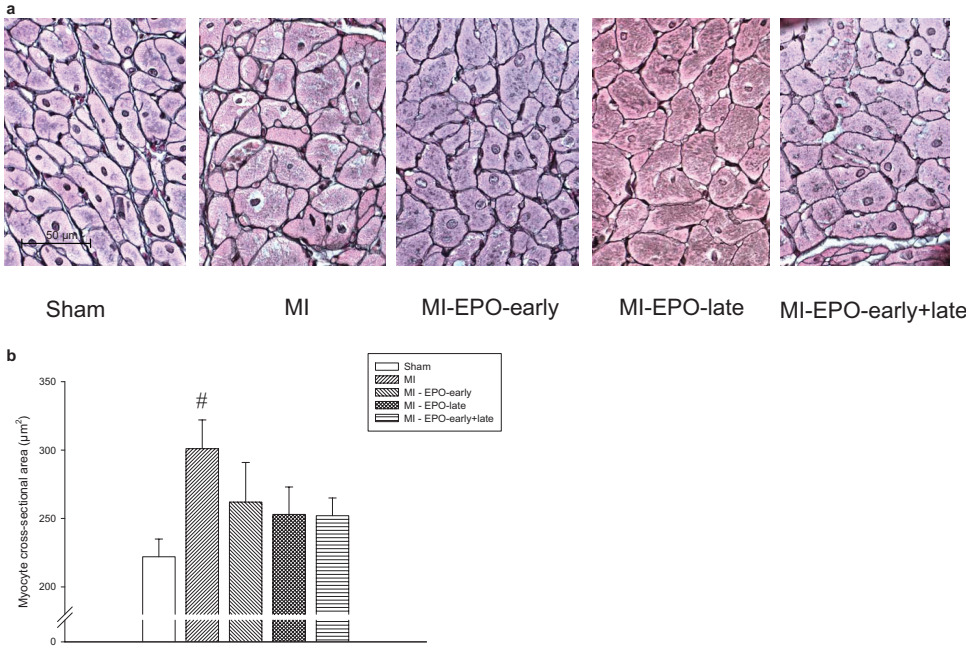


Figure 3a. Gomori stained sections in the LV viable wall of the five different groups, showing individual myocytes. **Figure 3b**. Bar graphs showing the actual measurements for the myocyte cross-sectional area in the different experimental groups. # $p<0.05$ vs. Sham.

Capillary density

Capillaries stained with lectin were clearly discernable in the myocardium. Figure 5A shows representative photomicrographs of the five different groups. Capillary density was significantly reduced in the MI group compared to the sham-group ($p<0.01$). EPO treatment in all three groups prevented the decrease in capillary density after induction of MI and restored it to sham values, as shown in Figure 5B (p=NS vs. sham). Furthermore, in the MI-EPO-late and MI-EPO-early+late groups, we observed a 39% and 48% increase in capillary to myocyte ratio, respectively (both $p<0.05$ vs. MI), whereas MI-EPO-early showed a clear trend ($p=0.05$ vs. MI) towards an increased capillary to myocyte ratio (figure 5C).

In order to relate LV functional parameters through a MHC-shift to increased capillarization, correlations were determined. We observed a strong correlation between capillary density and β-MHC expression ($r=-0.47$, $p<0.01$) and subsequently between β-MHC expression and cardiac contractility and relaxation, $r=-0.52$ and $r=0.61$ respectively (both $p<0.01$). Furthermore capillary density was correlated with myocardial contractility ($r=0.32$) and relaxation ($r=-0.37$; both $p<0.05$).

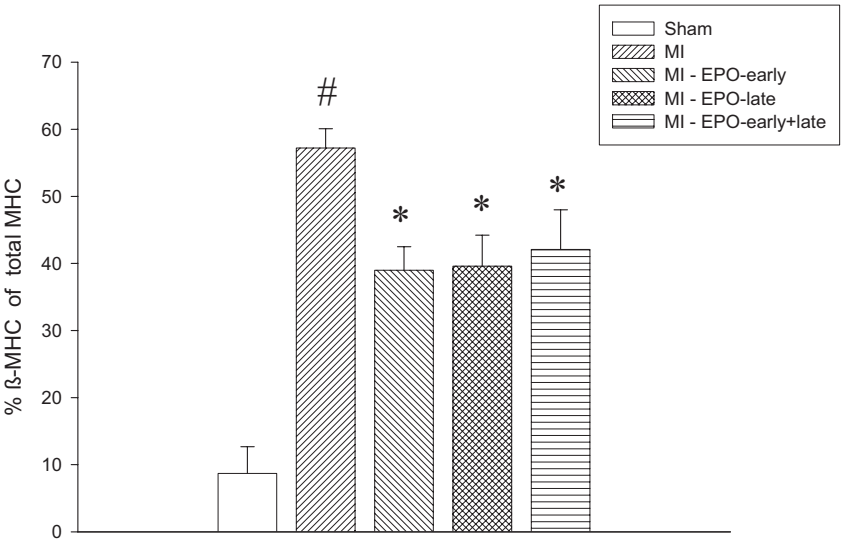


Figure 4. Effects of myocardial infarction and EPO treatment on β-MHC protein expression as a percentage of total MHC expression. * p<0.05 vs. MI, # p<0.01 vs. Sham.

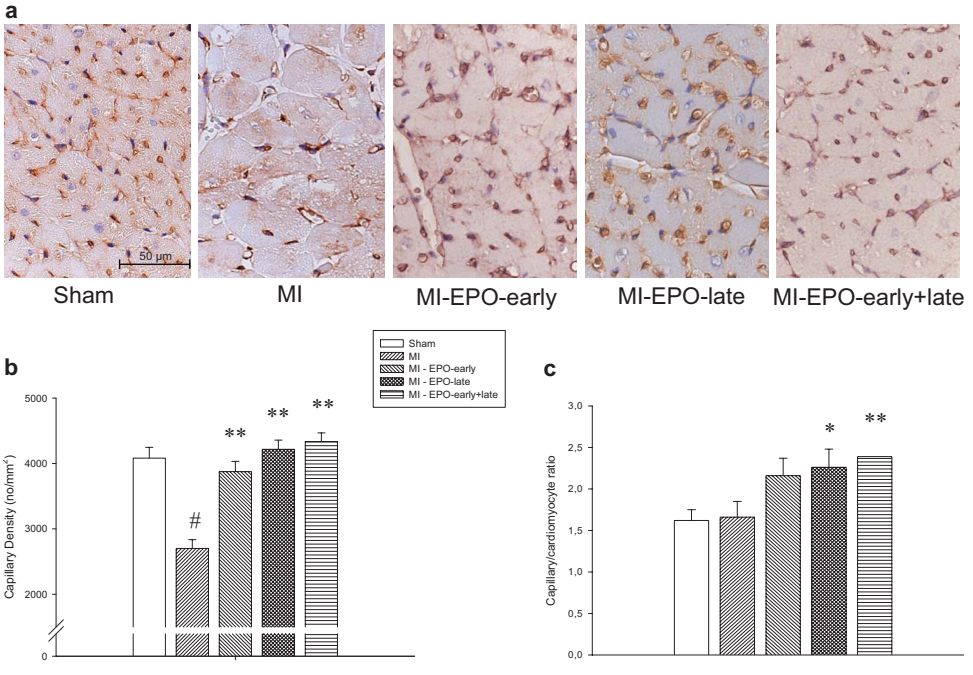


Figure 5a. Tissue sections stained with lectin in the viable free wall of the five different groups, showing individual capillaries. **Figure 5b**. Actual measurements for capillary density in number of capillaries per mm². **Figure 5c**. Bar graphs representing the capillary to myocyte ratio in the different treatment groups. * p<0.05 vs. MI, ** p<0.01 vs. MI, # p<0.01 vs. Sham.

Discussion

In the present study, the effects of EPO treatment in a rat model of post-MI heart failure were examined. To our knowledge, this study shows for the first time that EPO treatment initiated three weeks after induction of MI results in an improved cardiac function, as shown by a 17% increase of dLVP at 34% reduction in LVEDP and a 46% decrease in N-ANP levels. Furthermore, our data indicate that EPO restores capillary density to sham levels and increases the capillary to myocyte ratio, indicating neovascularization.

Myocardial structure and cardiac function

Previous studies already revealed that EPO has ancillary properties besides hematopoiesis. One of the first studies on EPO in the heart showed that EPO injected intraperitoneally for seven days, reduced cardiomyocyte loss by 50% after ischemia-reperfusion injury(25). These observations have been confirmed by others. Parsa *et al*. showed a 25% reduction in infarct size after 4 days of permanent occlusion of the left circumflex coronary artery in rabbits(12). A single dose of EPO at the onset of MI reduced infarct size, which was accompanied by reductions in LV size and an improved LV ejection fraction, measured by echocardiography, during eight weeks follow-up(26). Our results are in line with these findings; one single dose of EPO administered immediately after induction of MI, reduces infarct size by 30% and improves hemodynamics. Mechanisms behind this acute protective effect of EPO may be related to its anti-apoptotic effect. We and others showed that in the acute phase of MI, EPO markedly prevents cardiac cells from undergoing programmed cell death (apoptosis)(11;12;15;25). After MI, apoptosis is first observed in endothelial cells from small coronary vessels, spreading to the surrounding cardiomyocytes(27). Since the EPO-receptor is predominantly expressed on endothelial cells, preventing apoptosis in these cells may rescue the underlying myocardium(13). Recently, it has been postulated that cardiac fibroblasts may also play a role in the cardioprotective effects of EPO(15).

Although a single dose of EPO clearly improves cardiac performance, prolonged EPO treatment (MI-EPO-early+late) was associated with a further restoration of cardiac function. Mechanisms involved in this process are most likely distinct from its acute cardioprotective effect. This is clearly demonstrated by the finding that EPO treatment, initiated three weeks after MI, although not reducing infarct size, significantly improves cardiac function, reflected by a 17% increase of dLVP at 34% decrease in LVEDP, and restoring N -ANP levels to sham values. Since the effect of EPO treatment in this group could not be explained by infarct size reduction, other properties of EPO should be considered to elucidate the observed beneficial effects of EPO in heart failure.

Neovascularization

EPO has been shown to possess proangiogenic properties. As discussed above, the EPO-receptor is expressed on endothelial cells and EPO has been shown to stimulate the proliferation and migration of endothelial cells in vitro(6). Additional experiments in chick embryos demonstrated that EPO treatment results in angiogenesis similar to other well-known angiogenic cytokines(6). Furthermore, EPO induces vascular sprouting in a rat aortic ring model(28). In human cultured myocardial tissue, EPO stimulates capillary outgrowth comparable to VEGF(7). In a rodent model of hind-limb ischemia, EPO increases capillary density 1.6-fold(29). In a rat model of chronic renal failure, characterized by left ventricular hyper-

trophy and capillary deficiency, EPO treatment results only in a small non-significant increase in cardiac capillary density(30). In a rat model of stroke, EPO treatment, initiated 24 hours after infarction, enhances angiogenesis and improves neurological function, while it does not significantly influence infarct size. Our results suggest a similar effect of EPO in the heart. We find that EPO treatment restores capillary density to sham values and increases capillary to myocyte ratio, indicating actual capillary growth(24), which is more pronounced in the groups with prolonged EPO treatment.

To study the functional consequences of increased capillarization, we examined the expression of different MHC isoforms in heart tissue. Cardiomyocytes express both fast α-MHC and slow β-MHC isoforms, which differ on the basis of ATPase activity. Recently, it has been shown that expression of a small amount of α-MHC (~12%) in rat cardiomyocytes significantly increases power output, indicating that a small shift in MHC composition as we found in all EPO treated groups may improve contractility(31). Increased capillary density was significantly correlated with the percentage of β-MHC isoform as well as with myocardial function ($+dP/dt_{max}$ and $-dP/dt_{max}$), providing a link between neovascularization and functional effects of EPO.

The mechanism behind the effect of EPO on new blood vessel formation in the heart remains unknown. In general, stimulation of in situ endothelial cell proliferation or bone-marrow derived endothelial progenitor cells (EPCs) might play a role. Previous work showed that EPO effectively increases the amount of circulating EPCs(32), and significantly induces angiogenesis(29). Future experiments are needed to delineate the mechanism of EPO stimulated capillary growth.

Hematopoietic effect

Another important property of EPO that might be involved in the cardioprotective effect observed in our study, is its hematopoietic effect. Human recombinant erythropoietin increases the number of reticulocytes after administration to rats after 3-4 days with maximum after 8-11 days(33). In our study we observed significant hematocrit elevation one-week after a single dose of EPO. In the groups treated with multiple EPO doses, hematocrit remained significantly elevated throughout the experiment. The beneficial effects seen in these groups might thus, in part, be explained on the basis of increased oxygen-carrying capacity of blood. However, the effects of higher red blood cell mass on oxygen delivery is not straightforward, since elevated hematocrit may downregulate NO synthesis and thus impairs tissue blood flow(34). In the clinical setting, increasing the number of red blood cell mass by blood transfusion has been reported to improve outcome in elderly patients after acute MI(35). Nevertheless, this beneficial effect is only seen in patients with hematocrit <33%. On the other hand, reduction in the infarct size observed in the early treated groups, could not be attributed to the hematopoietic effect of EPO, since cell death and MI expansion occur mainly during the first 3 days after ischemic insult(26) and thus before significant hematocrit elevation.

Conversely, an increase in hematocrit may itself tend to deteriorate myocardial perfusion through adverse rheological effects. Elevated hematocrit levels (up to 80%) in polyglobulic mice, overexpressing EPO, enlarge cerebral infarct volumes and leukocyte infiltration after permanent occlusion of middle cerebral artery(36). Furthermore, EPO administration and consequent higher hematocrit has been associated with other adverse cardiovascular effects. Therapeutic levels of EPO may cause higher incidence of thrombosis (37) and could lead to blood pressure elevation(38). In the present study, rats repeatedly treated with EPO, show a

higher systolic blood pressure. This increase might be related to the improved cardiac function; however, the systolic blood pressure remained below the values observed in the sham operated group.

Clinical Implications

In clinical settings, EPO treatment has already been used to correct anemia in patients with CHF. Anemia is frequently observed in patients with CHF and related to increased morbidity and mortality(39;40). Furthermore, not only anemia, but also elevated endogenous EPO levels are independently associated with an impaired outcome in CHF(41). Normalization of hemoglobin levels in mild anemic patients with CHF has a positive effect on LV ejection fraction(42) and peak VO_2(43). In addition to correction of anemia, other non-hematopoietic effects of EPO may play a role in the improvement observed in patients with CHF treated with EPO.

Besides the treatment of anemia, EPO is currently under investigation for its neuroprotective properties. In the first clinical, randomized, proof-of concept trial, EPO was given to patients with ischemic stroke (44). EPO administration in high-doses (entire dose 100.000 IU/ given in three days) proved to be both safe and beneficial. Patients randomized to the EPO group showed significant improvement in clinical outcome parameters and a trend towards smaller infarct sizes.

However, chronic therapy with EPO is also associated with adverse effects related to hematocrit elevation, such as hypertension and tromboembolic complications. This could be overcome by using a lower dose of EPO, as shown by Bahlmann *et al*(45). In this study, low-dose of darbepoetin (0.1 μg/kg/week) rendered tissue protection in the kidneys even without raising hematocrit levels. The recently discovered non-hematopoietic derivates of EPO retaining tissue protection but without the undesired effects on hematopoiesis, may become another possibility for chronic administration (46).

Limitations

Several limitations of the present study have to be acknowledged. Although a clear increase in capillary density and capillary to myocyte ratio was observed, the improvement of cardiac function might also be related to other effects of EPO treatment. Since we did not perform sequential measurements of cardiac function, further studies would be needed to specifically denote the time-dependent effect of EPO treatment on attenuation of heart failure development.

We did not measure the direct myocardial perfusion and therefore, functional evidence of an improved perfusion remains unclear. However, we observed a clear correlation between capillary density and β-MHC expression and cardiac function. Furthermore, we used the Fisher's LSD post-hoc statistical test for analyzing our data, which does not control for multiple comparisons.

Conclusion

In summary, the present study demonstrates that EPO treatment in a rat model of heart failure improves cardiac function beyond its effect on infarct size reduction. This improvement could be explained by the increased capillary density and capillary to myocyte ratio, indicating formation of new blood vessels.

Acknowledgements

We thank Richard van Veghel for expert technical assistance. We further thank dr. Frans Boomsma for N-ANP measurements.

References

1. Anagnostou A, Liu Z, Steiner M *et al*. Erythropoietin receptor mRNA expression in human endothelial cells. Proc Natl Acad Sci U S A 1994;91:3974-8.

2. Chong ZZ, Kang JQ, Maiese K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. Circulation 2002;106:2973-9.

3. Van der Meer P., Voors AA, Lipsic E, van Gilst WH, van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. Eur Heart J 2004;25:285-91.

4. Wright GL, Hanlon P, Amin K, Steenbergen C, Murphy E, Arcasoy MO. Erythropoietin receptor expres sion in adult rat cardiomyocytes is associated with an acute cardioprotective effect for recombinant erythropoietin during ischemia-reperfusion injury. Faseb J 2004;18:1031-3.

5. De Boer RA, Pinto YM, van Veldhuisen DJ. The imbalance between oxygen demand and supply as a potential mechanism in the pathophysiology of heart failure: The role of microvascular growth and abnormalities. Microcirculation 2003;10:113-26.

6. Ribatti D, Presta M, Vacca A *et al*. Human erythropoietin induces a pro-angiogenic phenotype in cul tured endothelial cells and stimulates neovascularization *in vivo*. Blood 1999;93:2627-36.

7. Jaquet K, Krause K, Tawakol-Khodai M, Geidel S, Kuck K. Erythropoietin and VEGF exhibit equal angio genic potential. Microvasc Res 2002;64:326-33.

8. Wang L, Zhang ZG, Wang Y, Zhang RL, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 2004;35:1732-7.

9. Sakanaka M, Wen TC, Matsuda S *et al*. *In vivo* evidence that erythropoietin protects neurons from isch emic damage. Proc Natl Acad Sci U S A 1998;95:4635-40.

10. Siren AL, Fratelli M, Brines M *et al*. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A 2001;98:4044-9.

11. Lipsic E, van der Meer P, Henning RH *et al*. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. J Cardiovasc Pharmacol 2004;44:473-9.

12. Parsa CJ, Matsumoto A, Kim J *et al*. A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest 2003;112:999-1007.

13. Van der Meer P, Lipsic E, Henning RH *et al*. Erythropoietin improves left ventricular function and coro nary flow in an experimental model of ischemia-reperfusion injury. Eur J Heart Fail 2004;6:853-9.

14. Cai ZQ, Semenza GL. Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. Circulation 2004;109:2050-3.

15. Parsa CJ, Kim J, Riel RU *et al*. Cardioprotective effects of erythropoietin in the reperfused ischemic heart - A potential role for cardiac fibroblasts. J Biol Chem 2004;279:20655-62.

16. Van Kerckhoven R, van Veghel R, Saxena PR, Schoemaker RG. Pharmacological therapy can increase

capillary density in post-infarction remodeled rat hearts. Cardiovasc Res 2004;61:620-9.

17. Pfeffer JM, Pfeffer MA, Fletcher PJ, Braunwald E. Progressive ventricular remodeling in rat with myo cardial infarction. Am J Physiol 1991;260:H1406-H1414.

18. Wang Y, Zhang ZG, Wang L, Zhang RL, Chopp M. Erythropoietin enhances neurogenesis and angiogen esis in the brain and improves functional recovery after embolic stroke in the adult rat. Stroke 2004;35:239.

19. De Boer RA, Pinto YM, Suurmeijer AJ *et al*. Increased expression of cardiac angiotensin II type 1 (AT(1)) receptors decreases myocardial microvessel density after experimental myocardial infarction. Cardiovasc Res 2003;57:434-42.

20. Loot AE, Roks AJM, Henning RH *et al*. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. Circulation 2002;105:1548-50.

21. Nelissen-Vrancken HJMG, Kuizinga MC, Daemen MJAP, Smits JFM. Early captopril treatment inhibits DNA synthesis in endothelial cells and normalization of maximal coronary flow in infarcted rat hearts. Cardiovasc Res 1998;40:156-64.

22. Kalkman EAJ, Bilgin YM, vanHaren P, vanSuylen RJ, Saxena PR, Schoemaker RG. Determinants of coro nary reserve in rats subjected to coronary artery ligation or aortic banding. Cardiovasc Res 1996;32:1088-95.

23. Van der Velden J, Moorman AFM, Stienen GJM. Age-dependent changes in myosin composition cor relate with enhanced economy of contraction in guinea-pig hearts. J Physiol 1998;507:497-510.

24. Sladek T, Sladkova J, Kolar F *et al*. The effect of AT1 receptor antagonist on chronic cardiac response to coronary artery ligation in rats. Cardiovasc Res 1996;31:568-76.

25. Calvillo L, Latini R, Kajstura J *et al*. Recombinant human erythropoietin protects the myocardium from ischemia- reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci U S A 2003;100:4802-6.

26. Moon C, Krawczyk M, Ahn D *et al*. Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. Proc Natl Acad Sci U S A 2003;100:11612-7.

27. Scarabelli T, Stephanou A, Rayment N *et al*. Apoptosis of endothelial cells precedes myocyte cell apopto sis in ischemia/reperfusion injury. Circulation 2001;104:253-6.

28. Carlini RG, Reyes AA, Rothstein M. Recombinant-Human-Erythropoietin Stimulates Angiogenesis In-Vitro. Kidney Int 1995;47:740-5.

29. Heeschen C, Aicher A, Lehmann R *et al*. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 2003;102:1340-6.

30. Amann K, Buzello M, Simonaviciene A *et al*. Capillary/myocyte mismatch in the heart in renal failure a role for erythropoietin? Nephrol Dial Transplant 2000;15:964-9.

31. Herron TJ, McDonald KS. Small amounts of alpha-myosin heavy chain isoform expression significantly increase power output of rat cardiac myocyte fragments. Circ Res 2002;90:1150-2.

32. Bahlmann FH, DeGroot K, Duckert T *et al*. Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin - Rapid communication. Kidney International 2003;64:1648-52.

33. Eder H, Rosslenbroich B, Failing K. A dose-dependent effect of recombinant erythropoietin on the reticulocyte population of rats. Blut 1989;59:184-7.

34. Ruschitzka FT, Wenger RH, Stallmach T *et al*. Nitric oxide prevents cardiovascular disease and determines survival in polyglobulic mice overexpressing erythropoietin. Proc Natl Acad Sci U S A 2000;97:11609-13.

35. Wu WC, Rathore SS, Wang Y, Radford MJ, Krumholz HM. Blood transfusion in elderly patients with acute myocardial infarction. N Engl J Med 2001;345:1230-6.

36. Wiessner C, Allegrini PR, Ekatodramis D, Jewell UR, Stallmach T, Gassmann M. Increased cerebral in farct volumes in polyglobulic mice overexpressing erythropoietin. J Cereb Blood Flow Metab 2001;21:857-64.

37. Wolf RF, Gilmore LS, Friese P, Downs T, Burstein SA, Dale GL. Erythropoietin potentiates thrombus development in a canine arterio-venous shunt model. Thromb Haemost 1997;77:1020-4.

38. Roger SD, Baker LR, Raine AE. Autonomic dysfunction and the development of hypertension in pa tients treated with recombinant human erythropoietin (r-HuEPO). Clin Nephrol 1993;39:103-10.

39. McMurray JJ. What are the clinical consequences of anemia in patients with chronic heart failure? J Card Fail 2004;10:S10-S12.

40. Horwich TB, Fonarow GC, Hamilton MA, MacLellan WR, Borenstein J. Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. J Am Coll Cardiol 2002;39:1780-6.

41. Van der Meer P, Voors AA, Lipsic E, Smilde TDJ, van Gilst WH, van Veldhuisen DJ. Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure. J Am Coll Cardiol 2004;44:63-7.

42. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized con trolled study. J Am Coll Cardiol 2001;37:1775-80.

43. Mancini DM, Katz SD, Lang CC, LaManca J, Hudaihed A, Androne AS. Effect of erythropoietin on exer cise capacity in patients with moderate to severe chronic heart failure. Circulation 2003;107:294-9.

44. Ehrenreich H, Hasselblatt M, Dembowski C *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2002;8:495-505.

45. Bahlmann FH, Song R, Boehm SM *et al*. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. Circulation 2004;110:1006-12.

46. Fiordaliso F, Chimenti S, Staszewsky L *et al*. A nonerythropoietic derivative of erythropoietin protects the myocardium from ischemia-reperfusion injury. Proc Natl Acad Sci U S A 2005;102:2046-51.

Chapter 7

Low-dose erythropoietin treatment preserves cardiac function and mobilizes endothelial progenitor cells in rats with ischemic heart failure

Erik Lipšic, Peter van der Meer, Pim van der Harst, B. Daan Westenbrink, Johan Koster, Gideon J. du Marchie Servaas, Dirk J. van Veldhuisen, Regien G. Schoemaker, Wiek H. van Gilst

Submitted

Abstract

Background: The hematopoietic hormone erythropoietin (EPO) is involved in postnatal neovascularization and was shown to enhance endothelial progenitor cells (EPCs) mobilization from bone marrow. We hypothesized that low-dose EPO treatment, not leading to hematocrit increase, would mobilize EPCs, increase capillarization and preserve cardiac function in a post-myocardial infarction (MI) heart failure model.

Methods and Results: Rats were either subjected to left coronary artery ligation or sham surgery. Rats with MI were left untreated (MI group) or long-acting EPO analogue darbepoetin treatment was started 3 weeks after MI in a low-dose (0.4 μg/kg/3 weeks, MI-EPO-low) or high-dose (40 μg/kg/3 weeks, MI-EPO-high). Echocardiography was used to measure the cardiac function during follow-up. After 9-weeks, hemodynamics, number of EPCs and capillary density were measured. Hematocrit increased in the MI-EPO-high, but not in MI-EPO-low group ($p<0.01$). Both high- and low-dose EPO treatment resulted in preservation of left ventricular systolic function during follow-up, and improved cardiac contractility (dP/dt_{max}) and relaxation (dP/dt_{min}) at 9-weeks, compared to MI group (all $p<0.05$). In addition, in EPO-treated groups the number of circulating EPCs was significantly increased (MI-EPO-high: 82 [66-147] and MI-EPO-low: 67 [55-107] vs. MI: 23 [19-33] cells/high-power field; both $p<0.01$). This was associated with a 33% increase in capillary density in MI-EPO-high ($p<0.01$) and 20% in MI-EPO-low ($p<0.05$), compared to MI group.

Conclusions: EPO treatment preserves cardiac function in post-MI heart failure, even in doses not affecting hematocrit level. This is associated with raised number of circulating EPCs and an increased capillary density.

Introduction

The classical role of erythropoietin (EPO) is related to its hematopoietic effects. Production of EPO in the kidney is upregulated in response to lower blood oxygen levels. EPO acts as a major regulator of erythropoiesis, by increasing survival and promoting proliferation of erythroid precursor cells.

Recently, several non-hematopoietic effects of EPO have been reported. EPO was shown to render vascular protection in various experimental models of ischemia, including stroke and myocardial infarction [1]. During *ex-vivo* ischemia-reperfusion in isolated rat hearts, EPO perfusion improved cardiac function and increased coronary flow [2,3]. Acute, systemic treatment with EPO, in a rodent ischemia-reperfusion model, substantially reduced infarct size and decreased myocardial apoptosis [4], even when EPO was administered after reperfusion [5,6].

Another important pleiotropic effect of EPO is the promotion of postnatal angiogenesis and vasculogenesis. Previously, expression of EPO-receptor on endothelial cells has been reported [3] and EPO was shown to stimulate endothelial cell proliferation and differentiation into vascular structures [7]. Recent studies demonstrated that circulating endothelial progenitor cells (EPCs) are involved in neovascularization and differentiate into endothelial cells in situ [8,9]. In a study of Heeschen *et al.*, EPO was shown to increase the number of circulating EPCs in the peripheral blood [10]. Moreover, treatment with EPO significantly increased inflammation- and ischemia-induced neovascularization [10]. In heart failure patients, chronic EPO treatment was associated with an increase in the adhesive and proliferative properties of circulating EPCs [11].

Recently, we assessed the effects of EPO treatment in a rat model of post-myocardial infarction (MI) heart failure. In this study, prolonged high-dose EPO treatment was associated with improved cardiac function and restored capillary density to sham values, indicating actual capillary growth [12].

However, because high-dose EPO treatment is also associated with increased hematocrit values, the observed beneficial effects may, at least to some extend, be related to the increased oxygen-carrying capacity of blood. In the clinical situation, repeated therapy with high-dose EPO could lead to unwanted elevation of hematocrit, coupled with higher risk for thrombosis and hypertension. In addition, the existence of different receptors that mediate the effects of EPO in distinct tissues [13], may suggest also different dose-response relationships for the various protective effects.

Therefore, we studied the effects of high- and low-dose EPO treatment on cardiac function over time, EPCs mobilization and neovascularization in a post-MI heart failure.

Methods

Animals

We used male Sprague Dawley rats weighing 270-330 g (Harlan, Zeist, The Netherlands). Animals were fed ad libitum, and housed in groups of four to five rats, according to institutional rules with 12:12 hours light-dark cycles. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Design of the study

Rats were either subjected to left coronary artery ligation (n=63) or sham surgery (n=11). Rats with MI were allocated to 3 groups: control (MI) and two EPO treatment groups with different dosages of long-acting EPO analogue darbepoetin: 40 μg/kg (MI-EPO-high) and 0.4 μg/kg (MI-EPO-low). Darbepoetin-alpha (Aranesp, Amgen Inc., Thousands Oaks, CA, USA) was administered intraperitoneally, once every three weeks, starting three weeks after the coronary artery ligation, hence after the development of post-MI heart failure. Control (MI) and SHAM rats received corresponding injections of saline. The allocation of MI rats to different groups was based on echocardiography performed at week 3 (before the start of the therapy), groups were matched based on left ventricular (LV) end-diastolic diameter and LV fractional shortening.

The high dose of darbepoetin was based on our previous study [12], demonstrating increased neovascularization in this model, together with significant elevation of hematocrit levels. To avoid the effect of EPO treatment on hematocrit we included a low-dose EPO group, with 100-times lower darbepoetin dosage (0.4 μg/kg/3 weeks), which in our pilot experiment did not cause elevation of hematocrit (data not shown). Hematocrit was measured at baseline and at week 3, 4, 6 and 9 after surgery.

Myocardial infarction model

This model has been described previously [14]. Briefly, rats were anesthetized with 2.5% isoflurane and placed on a heating pad (37^{0}C). Animals were intubated and mechanically ventilated (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands; frequency 90/min) using room air enriched with 1.0 l/min oxygen. After left-side thoracotomy, MI was induced by ligating the proximal portion of the left coronary artery, beneath the left atrial appendage. In sham operated rats, the same surgery was performed, without ligating the suture.

Echocardiographic measurements

Cardiac function was assessed by echocardiography (Vivid 7, GE Healthcare, Chalfont St. Giles, United Kingdom; equipped with a 10-Mhz phase array linear transducer) at baseline (before coronary artery ligation), at week 3 (before start of the therapy), 6 and 8. The echocardiographic measurements were performed under general anesthesia with 2.5% isoflurane, by two researchers blinded for the treatment allocation. The rats were placed on a heating pad (37^{0}C) and transducer was applied parasternally to the shaved chest wall. The transducer was maneuvered to obtain both 2-dimensional (2D) images in parasternal long-axis and short-axis view and 2-D guided M-mode tracing. Long axis views were obtained, ensuring that the mitral and aortic valves and the apex were visualized. Short axis views were recorded at the level of mid-papillary muscles. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from the M-mode and calculated as an average from short- and long-axis view. LV fractional shortening (FS %) was calculated as FS= (LVEDD-LVESD)/LVEDD x 100. LV ejection fraction (EF %) was calculated using the Teichholz method of estimated LV volumes [15].

Hemodynamic measurements

After nine weeks rats were anesthetized as described above. Microtip pressure transducer (Millar Instr. Inc., Houston, Texas, USA) was inserted into the left ventricular cavity via the right carotid artery. After a 3-min period of stabilization, heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and developed left ventricular pressure (dLVP=LVSP-LVEDP) were measured. As indices of contractility and relaxation, the maximal rates of increase and decrease in LVP (dP/dt_{max} and dP/dt_{min}) were determined. The catheter was retracted into the aortic arch and arterial systolic and diastolic blood pressures (SBP, DBP) were recorded.

Infarct size

After hemodynamic measurements, hearts were rapidly excised and weighed. Mid-papillary slices were prepared for immunohistochemistry. Slices were fixed in 4% paraformaldehyde and paraffin-embedded. Infarct size was determined by planimetry at mid-ventricular levels in transverse slices on picrosirius red/fast green–stained sections. Infarct size was expressed as the percentage of scar length to total left ventricular circumference, as described previously [16,17].

Blood derived endothelial progenitor cells

Full blood was collected in heparine tubes (17 IU/ml). Mononuclear cells were isolated using Histopaque-1083 (Sigma Chemical, St. Louis, MO, USA) according to the instructions as supplied by the manufacturer and counted on microcellcounter (Sysmex F-800, Toa Medical Electronics, Kobe, Japan). Isolated mononuclear cells (1×10^6) were seeded into fibronectin-

precoated 24-well plates (BD BioCoat, Bedford, MA, USA) in EndoCult medium (StemCell Technologies, London, UK) supplemented with Penicillin (100U/mL) and Streptomycin (100µg/mL). After 6 days, adherent cells were washed with medium, incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL (10µg/mL Dil AcLDL; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 12 hours, fixed with 1% paraformaldehyde for 10 minutes, and counterstained with fluorescein isothyiocyanate-labeled *Griffonia (bandeiraea) simplicifolia* lectin I, isolectin B_4 (lectin, 10 µg/mL; Vector Laboratories, Burlingame, CA, USA). Images were captured by an inverted fluorescence microscope (Axiovert 135 M, Carl Zeiss, Jena, Germany). Cells double positive for DiI AcLDL and lectin staining were considered EPCs and counted in high-power fields with the co-localization analysis (Image-Pro Plus for Windows, version 4.5.0.29). For every rat an average number of circulating EPCs was calculated from 4-5 high-power fields.

Capillary density

To visualize the capillaries in the myocardium of the LV free wall, endothelial cells were stained with biotin-labeled Lectin GSL (1:100; Sigma-Aldrich, St. Louis, Missouri, USA), as previously described [14]. Since lectins stain not only capillaries but other vessels as well, a size criterion of 10 µm was used to exclude small arterioles and venules. Image analysis (Image-Pro Plus for Windows, version 4.5.0.29) was used to measure capillary density, calculated as the number of capillaries per tissue area (mm^2). The measured total tissue area was corrected for the remaining interstitial space.

Statistical analysis

Data are presented as mean ± SEM, or as median ± IQR (25^{th} and 75^{th} percentile) depending on their distribution. Differences among groups were tested using one-way analysis of variance, followed by LSD post-hoc analysis if normally distributed, and by Kruskal-Wallis test if skewed distributed. Correlation analysis was performed with Spearman's correlation test. All reported probability values were 2-tailed, and a p-value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS version 11.0.

Results

Mortality and general characteristics

Overall 24-hour mortality following the MI was 41%. No mortality was observed in sham-operated rats. Five additional MI rats died during the 9-week long follow-up (2 in MI group, 1 in MI-EPO-high and 2 in MI-EPO-low group).

General characteristics after nine weeks are shown in table 1. Two rats (1 in MI and 1 in MI-EPO-high group) had infarct size < 25%. These were excluded from further analysis. LV-infarct size (% of LV) was comparable between all MI groups (table 1). Body weight was significantly higher only in the MI-EPO-low group (table 1).

The heart weight (HW) to body weight ratio was significantly increased in the rats with MI compared to the sham rats (all $p<0.05$; table 1). A lower HW to body weight compared to MI group was observed in MI-EPO-high and MI-EPO-low groups (both $p<0.05$), suggesting diminished cardiac hypertrophy after MI.

Table 1. Characteristics of the experimental groups

	Sham	MI	MI-EPO-high	MI-EPO-low
General				
n	11	10	10	9
Infarct size	-	45.5±3.6	46.7±1.5	50.0±3.2
Hemodynamics				
Heart rate (bpm)	321±6	318±8	339±7	312±7
LVSP (mmHg)	130±3	104±6†	119±3*§	115±4†
LVEDP (mmHg)	9.8±0.6	23.5±3.7†	15.7±2.0‡	18.8±2.3*
SBP (mmHg)	127±3	102±5†	115±3*‡	113±4*
DBP (mmHg)	81±2	73±3*	83±3§	80±2
Body/organ weight				
BW (g)	410±6	402±12	416±8	442±8*§
Heart weight/BW (mg/g)	4.5±0.1	6.0±0.3†	5.3±0.1†‡	5.3±0.3*‡
Hematocrit				
Baseline (%)	48±0.9	46±0.7	48±0.6	48±0.7
Week 3 (%)	49±0.8	50±0.7	49±0.4	50±0.5
Week 4 (%)	51±0.9	51±0.8	64±1.1†§	52±0.7
Week 6 (%)	49±0.8	50±1.0	56±0.6†§	51±0.5
Week 9 (%)	44±0.6	44±0.6	53±1.4†§	42±0.9

Data are presented as mean ± SEM; n indicates number of animals. bpm, beats per minute; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; BW, bodyweight. *p<0.05; †p<0.01 vs. Sham; ‡p<0.05, §p<0.01 vs. MI.

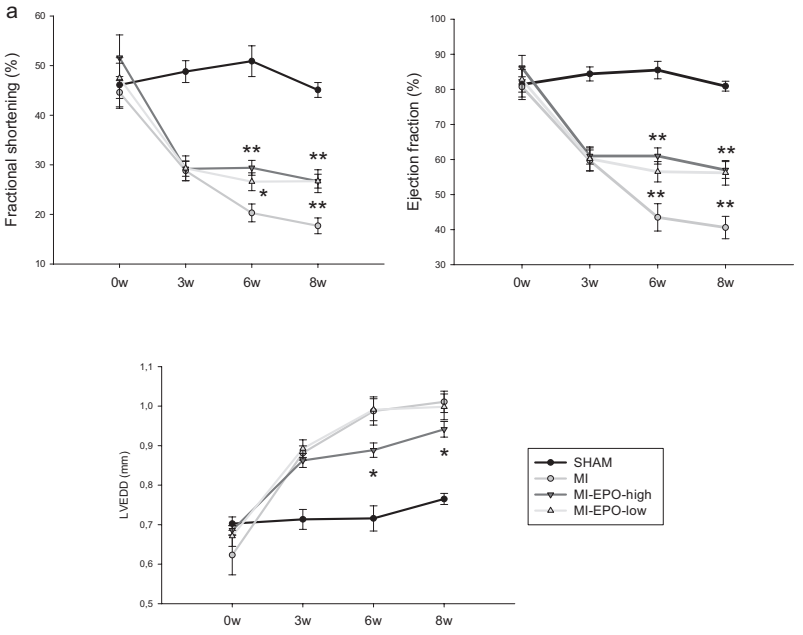
Low-dose EPO does not increase hematocrit

The changes of the hematocrit throughout the experiment are shown in table 1. Only in the MI-EPO-high group the treatment with high-dose EPO led to significant increase in hematocrit levels, which persisted throughout the experiment. Importantly, hematocrit levels in MI-EPO-low group were similar to those of MI and sham groups.

Low-dose EPO improves cardiac function

Serial echocardiographic parameters are presented in figure 1. Baseline echocardiographic measurements of the LV size and function did not differ among groups before the coronary artery ligation. Induction of MI led to a significant enlargement of LVEDD and deterioration of LV performance (fractional shortening and ejection fraction) at 3 weeks. During treatment follow-up, LV performance (both EF and FS) was significantly better in both MI-EPO-high and MI-EPO-low group, as compared to control. While the deterioration of LV function progressed gradually in the MI group throughout the remaining 6 weeks, it remained stable after the initiation of EPO treatment in both the MI-EPO-high and MI-EPO-low group. Treatment with high-dose EPO prevented the progression of LV dilation (LVEDD), when compared to MI group at week 6 and 8 (both $p<0.05$). On the contrary low-dose EPO treatment did not decelerate the LV dilation after the MI.

To further evaluate the hemodynamic profile of all experimental groups, invasive pressure



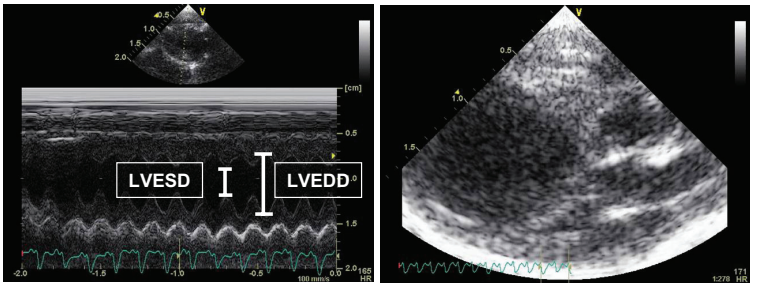


Figure 1a. Changes in echocardiographic indices of LV size and function during 8-weeks follow-up after coronary artery ligation. LVEDD indicates left ventricular end-diastolic diameter. * p<0.05 vs. MI, ** p<0.01 vs. MI. **1b**. M-mode echocardiographic picture of a sham rat with calculation of LV size (left picture); 2D-picture of long axis in a rat after MI (right picture); LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter.

measurements were performed 9 weeks after the surgery, directly before the rats were sacrificed. Myocardial contractility (dP/dt_{max}) and myocardial relaxation (dP/dt_{max}) were both impaired in all MI groups compared to the sham group (all $p<0.05$). Both low- and high-dose EPO treatments resulted in better contractility and relaxation compared to MI (all $p<0.05$; figure 2). LVSP and developed LVP (dLVP) were both decreased in all MI groups compared to sham operated rats ($p<0.05$ for all). MI-EPO-high showed a significantly higher LVSP (table 1) and dLVP (figure 2), compared to MI (both $p<0.01$). Low-dose of EPO resulted in a 17% higher dLVP ($p<0.05$; figure 2), and a trend towards elevation of LVSP, compared to control group ($p=0.07$; table 1). LVEDP was elevated in MI-group compared to sham operated rats ($p<0.01$; table 1). In the MI-EPO-high group the LVEDP was 34% ($p<0.05$) and in the MI-EPO-low group 20% (p=NS) lower, compared to MI. Systolic blood pressure (SBP)

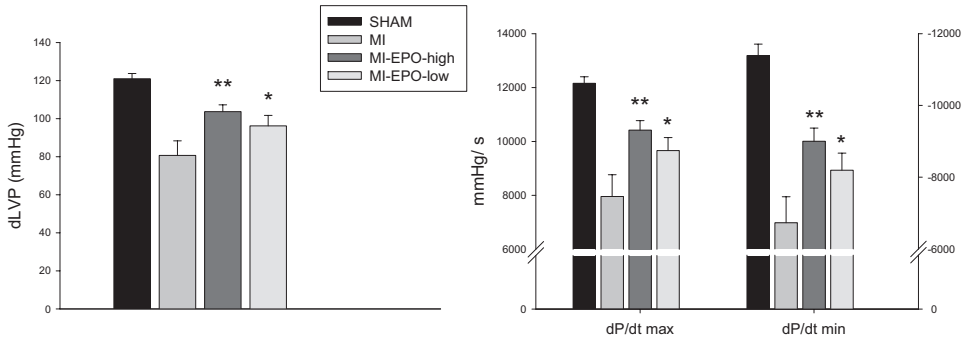


Figure 2. Effects of myocardial infarction and different doses of EPO treatment on hemodynamic parameters. dLVP indicates developed left ventricular pressure; dP/dt_{max} and dP/dt_{min}, maximal rate of increase and decrease of ventricular pressure, respectively. * $p<0.05$ vs. MI, ** $p<0.01$ vs. MI.

was significantly lower in all MI groups compared to sham. SBP was significantly higher in MI-EPO-high, compared to MI group ($p<0.05$). The diastolic blood pressure (DBP) was lower compared to sham only in the control MI group ($p<0.05$). In both MI-EPO-high and MI-EPO-low, DBP was similar to that of sham group. No significant differences were observed in heart rate.

EPO dose-dependently increases circulating endothelial progenitor cells

Induction of MI led to a decrease in the number of peripheral EPCs, at 9 weeks (23.2 [18.8-32.8] in MI-group vs. 57.2 [35.0-59.8] per high-power field in sham group; $p<0.05$). Treatment with both high- and low-dose EPO resulted in a 3.6- and 2.9-fold increase in the number of circulating EPCs, respectively, compared to MI group (both $p<0.01$; figure 3).

Low- and high-dose EPO treatment increases capillary density

Capillaries stained with lectin were clearly discernable in the myocardium. Figure 4B shows representative photomicrographs of the four different groups. Capillary density was significantly reduced in MI compared to sham-group ($p<0.01$). High-dose EPO treatment prevented the decrease in capillary density after induction of MI and restored it to sham values, as shown in Figure 4A ($p=$NS vs. sham). In this group (MI-EPO-high) we observed a 33% increase in capillary density compared to MI group ($p<0.01$). Treatment with low-dose EPO resulted in a 20% higher capillary density ($p<0.05$). The differences between MI-EPO-high and MI-EPO-low group were not statistically significant.

In order to relate mobilization of EPCs to increased capillarization, correlations were determined. We observed a positive correlation between the number of capillaries per mm^2 and the number of peripheral EPCs ($r=0.32$; $p<0.05$), which was even stronger in the rats subjected to coronary artery ligation ($r=0.59$; $p<0.01$).

Discussion

In the present study, we examined the effects of high- and low-dose EPO treatment on cardiac function in a post-MI heart failure model. We show that both treatments attenuate post-MI remodeling and improve cardiac function. Furthermore, this is associated with mobilization of endothelial progenitor cells and increased capillary density. Recently, numerous experimental studies have demonstrated important non-hematopoietic functions of EPO, such as protection against ischemic injury in various tissues. In the first *in vivo* study on the EPO effects in the heart, Calvillo *et al* [18], employed a rat model of coronary ischemia-reperfusion. Administration of EPO (5.000 IU/kg/day) for seven consecutive days after reperfusion reduced the loss of cardiomyocytes by 50%, an extent sufficient to normalize hemodynamic function. However, the hematocrit increased by 20-30% by the end of the study, and to some extent, such a change could lead to improved cardiac function merely by improving the delivery of oxygen. In a study by Parsa *et al*. [19], administration of high-dose EPO (5.000 IU/kg) reduced the infarct size both after ischemia-reperfusion, as well as permanent coronary occlusion, but resulted in a 20% increase in hematocrit by day 4. Mechanism behind this acute cardioprotective effect of EPO is probably related to inhibition of apoptosis [5,20].

In clinics, current therapy in patients after MI is focused on prevention of ventricular remodeling and development of heart failure. Myocardial regeneration may offer possibilities that could improve cardiac function in these patients [21]. Although cardiomyocytes proliferation after ischemic injury seems limited, the formation of new vessels in the non-infarcted part of the ventricle could lead to an improvement of function and attenuation of ventricular remodeling [22,23].

In addition to its anti-apoptotic properties during acute ischemic injury, EPO was recently shown to mobilize EPCs from the bone marrow, which was associated with neovascularization (vasculogenesis) of ischemic tissue [10]. EPO has also been shown to stimulate proliferation of endothelial cells in situ (angiogenesis) [24]. In a rat aortic ring model, EPO induced vascular sprouting [25]. In human cultured myocardial tissue, EPO stimulated capillary outgrowth comparable to VEGF [26].

The effect of EPO on the formation of new vessels has also been observed in an experimental model of stroke. EPO treatment, initiated 24 hours after induction of stroke, enhanced neovascularization and improved neurological function, while it did not significantly influence infarct size [27]. In this study, high-dose EPO treatment for seven consecutive days (5.000-10.000 IU/kg/day) increased the density of microvessels at the stroke boundary (ischemic penumbra), but it also resulted in a 44% increase in hematocrit level.

We addressed this issue in the heart, evaluating the effect of EPO treatment on new vascular formation in an experimental heart failure model [12]. Rats were subjected to coronary artery ligation and therapy with high-dose EPO analogue darbepoetin (40 µg/kg/3 weeks) was initiated 3-weeks post-MI. Although not reducing infarct size, EPO treatment significantly improved cardiac function. This improvement was coupled to increased capillary density and capillary-to-myocyte ratio, indicating neovascularization. Furthermore, these beneficial effects were also associated with increased percentage of alpha-MHC (myosine-heavy chain) isoforms, a molecular marker of enhanced myocardial contractility.

However, the dosages used in the previous studies, when applied to clinical situation, could cause EPO overdose which may lead to hypertension, seizures, vascular thrombosis and death, possibly related to abruptly increased hematocrit values [28]. This could be of potential concern

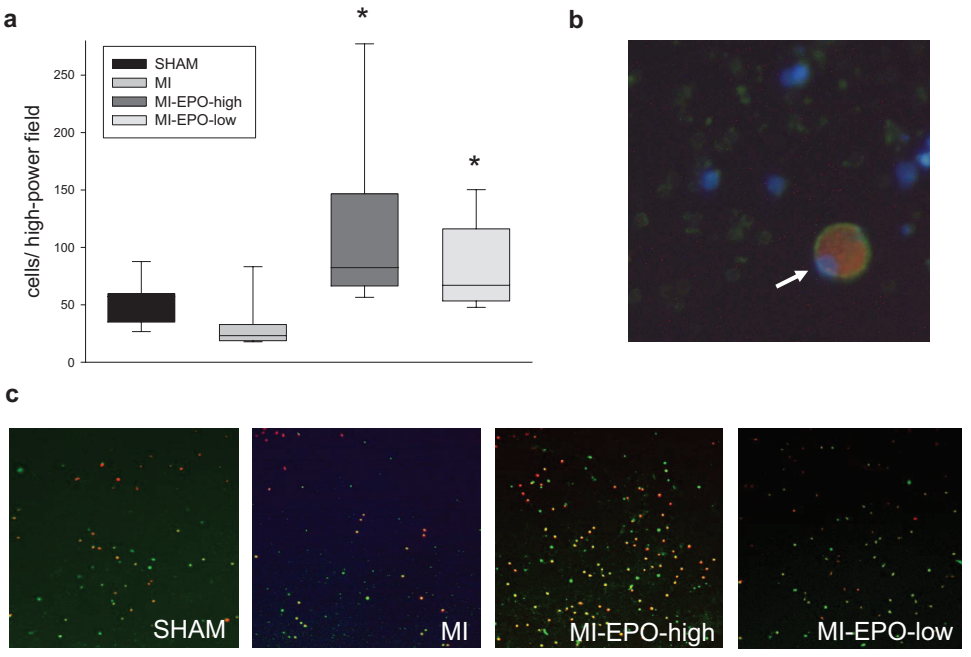


Figure 3. Effects of EPO treatment on number of circulating EPCs. **a**, Graphic representation of number of EPCs. The boxplots show the median with 25-75% range, the error bars show 10-90% range of the number of EPCs per high-power field. *p<0.01 vs. MI. **b**, Endothelial progenitor cell under high magnification (white arrow), positively stained for DiI AcLDL (red cytoplasm) and lectin (green cytoplasm), including DAPI nuclear staining (blue). **c**, Representative microscopic fields from all experimental groups, with double stained (green+red) positive EPCs.

in patients with already elevated cardiovascular risk.

In our study, we chose a low-dose EPO treatment to avoid elevation of hematocrit, and consequently the changes in rheology and oxygen-binding capacity of the blood, which could both influence our results. We also included a group with a high EPO dose (MI-EPO-high), leading to a marked hematocrit elevation, which effects on cardiac function in post-MI heart failure were already established [12]. The low dose of EPO was two order of magnitude lower compared to high dose (40 vs. 0.4 μg/kg/3 weeks). We did not observe any differences in hematocrit levels between the MI-EPO-low and both saline treated groups (MI and sham), while in MI-EPO-high the hematocrit remained significantly elevated during the treatment follow-up.

Treatment with low-dose EPO prevented the deterioration of LV function over time, as assessed by LV ejection fraction and fractional shortening, serially evaluated by echocardiography. However, low-dose EPO, as opposed to high-dose, did not avoid the progression of LV dilation. While this could mean a dose-dependent effect of EPO, bigger infarct size in MI-EPO-low group, although not significantly different from other groups, could have averted the beneficial effects of EPO on LV geometry. Low-dose EPO treatment thus, in spite of advanced LV dilation, improves contractile properties of the non-infarcted part of the myocardium. Importantly, this effect was also confirmed by invasive hemodynamic measurements at the end of the study. Although the treatment with high-dose resulted in noticeable improved cardiac function, low-dose treatment also caused a significant enhancement of developed LVP, together with contractility and relaxation indices of the LV.

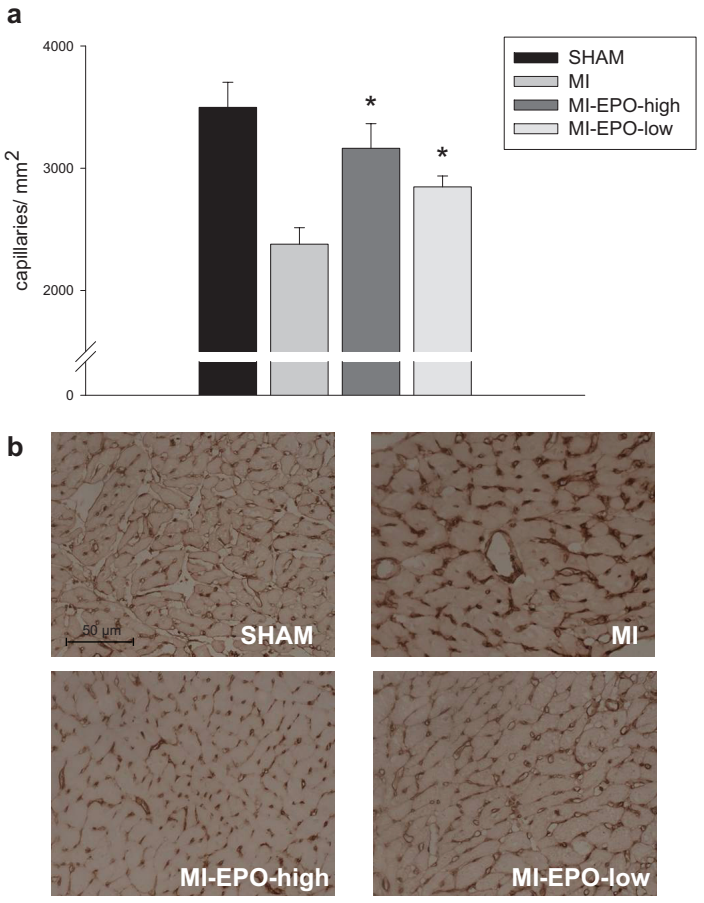


Figure 4. Effect of EPO treatment on capillary density. A, Actual measurements of capillary density in number of capillaries per mm². *p<0.05 vs. MI. B, Tissue sections with lectin in the viable free wall of the four different groups, showing individual capillaries.

A lower heart weight to body weight ratio in MI-EPO-high and MI-EPO-low group compared to control also indicates attenuation of cardiac hypertrophy by both EPO treatment regimens and beneficial effects on post-MI cardiac remodeling.

We confirmed the findings of our previous experiments, that high-dose EPO treatment increases capillary density, promoting neovascularization after ischemic injury in the heart. In addition, low-dose EPO, although to a lesser extent, increased the capillary density by 20%. Both high- and low-dose EPO treatment increased the number of circulating EPCs. This is line with findings of Bahlmann *et al.*[29], who found that darbepoetin stimulates bone marrow-derived EPCs at doses that do not increase the hematocrit. Moreover, the increased number of EPCs was significantly correlated with the amount of capillaries per mm², providing an interesting link between EPCs mobilization and increased capillarization in the heart.

Application of lower doses of EPO was also shown to confer vascular and tissue protection in the kidney [30]. Low-dose darbepoetin treatment in a rat remnant kidney model improved the survival, ameliorated endothelial damage and preserved renal function, without increase

in hematocrit levels. Another option to avoid the negative effects of chronic EPO therapy on hematocrit values, could be the use of recently discovered nonhematopoietic derivates of EPO, retaining the tissue protecting property, without undesired effect on hematopoiesis [31]. The possibility to separate the hematopoietic and tissue-protective effect could be explained through interaction of EPO with different receptors in various tissues [32]. However, the role of these receptors in EPCs stimulation is unknown, and thus the regenerative capacity of the novel EPO derivates may be attenuated by reduced mobilization of EPCs.

In summary, low-dose EPO treatment preserves cardiac function in post-MI heart failure. This is associated with raised number of circulating EPCs and an increased capillary density. Although time-limited treatment with high-dose EPO may be beneficial and safe during acute ischemic injury, if prolonged therapy is required (heart failure), drug regimens using low-dose EPO may be more suitable in avoiding the adverse effects of the treatment.

Acknowledgements

We thank Azuwerus van Buiten, Maaike Goris and Liza Wong for expert technical assistance.

References

1. Van der Meer P., Voors AA, Lipsic E, van Gilst WH, van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. *Eur Heart J* 2004;25:285-291.

2. Cai Z, Semenza GL. Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. *Circulation* 2004;109:2050-2053.

3. Van der Meer P, Lipsic E, Henning RH, De Boer RA, Suurmeijer AJ, Van Veldhuisen DJ, Van Gilst WH. Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury. *Eur J Heart Fail* 2004;6:853-859.

4. Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, Koch WJ. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003;112:999-1007.

5. Lipsic E, van der Meer P, Henning RH, Suurmeijer AJ, Boddeus KM, Van Veldhuisen DJ, Van Gilst WH, Schoemaker RG. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. *J Cardiovasc Pharmacol* 2004;44:473-479.

6. Parsa CJ, Kim J, Riel RU, Pascal LS, Thompson RB, Petrofski JA, Matsumoto A, Stamler JS, Koch WJ. Cardioprotective effects of erythropoietin in the reperfused ischemic heart: a potential role for cardiac fibroblasts. *J Biol Chem* 2004;279:20655-20662.

7. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood* 1999;93:2627-2636.

8. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Isch emia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nature Medicine* 1999;5:434-438.

9. Shi Q, Rafii S, Wu MHD, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, Hammond WP. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 1998;92:362-367.

10. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003;102:1340-1346.

11. George J, Goldstein E, Abashidze A, Wexler D, Hamed S, Shmilovich H, Deutsch V, Miller H, Keren G, Roth A. Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. *Cardiovasc Res* 2005.

12. Van der Meer P, Lipsic E, Henning RH, Boddeus K, van der Velden J, Voors AA, Van Veldhuisen DJ, Van Gilst WH, Schoemaker RG. Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction. *J Am Coll Cardiol* 2005;46:125-133.

13. Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, Latini R, Xie QW, Smart J, Su-Rick CJ, Pobre E, Diaz D, Gomez D, Hand C, Coleman T, Cerami A. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc Natl Acad Sci U S A* 2004;101:14907-14912.

14. Van Kerckhoven R, van Veghel R, Saxena PR, Schoemaker RG. Pharmacological therapy can increase capillary density in post-infarction remodeled rat hearts. *Cardiovasc Res* 2004;61:620-629.

15. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determina tions: echocardiographic-angiographic correlations in the presence of absence of asynergy. *Am J Cardiol* 1976;37:7-11.

16. Loot AE, Roks AJM, Henning RH, Tio RA, Suurmeijer AJH, Boomsma F, van Gilst WH. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation* 2002;105:1548-1550.

17. Nelissen-Vrancken HJMG, Kuizinga MC, Daemen MJAP, Smits JFM. Early captopril treatment inhibits DNA synthesis in endothelial cells and normalization of maximal coronary flow in infarcted rat hearts. *Cardiovasc Res* 1998;40:156-164.

18. Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, Salio M, Cerami A, Brines M. Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc Natl Acad Sci U S A* 2003;100:4802-4806.

19. Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, Koch WJ. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003;112:999-1007.

20. Moon C, Krawczyk M, Ahn D, Ahmet I, Paik D, Lakatta EG, Talan MI. Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. *Proc Natl Acad Sci U S A* 2003;100:11612-11617.

21. Lee MS, Makkar RR. Stem-cell transplantation in myocardial infarction: a status report. *Ann Intern Med* 2004;140:729-737.

22. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 2005;115:572-583.

23. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004;428:664-668.

24. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neo vascularization *in vivo*. *Blood* 1999;93:2627-2636.

25. Carlini RG, Reyes AA, Rothstein M. Recombinant-Human-Erythropoietin Stimulates Angiogenesis In-Vitro. *Kidney Int* 1995;47:740-745.

26. Jaquet K, Krause K, Tawakol-Khodai M, Geidel S, Kuck K. Erythropoietin and VEGF exhibit equal angio genic potential. *Microvasc Res* 2002;64:326-333.

27. Wang Y, Zhang ZG, Wang L, Zhang RL, Chopp M. Erythropoietin enhances neurogenesis and angiogen esis in the brain and improves functional recovery after embolic stroke in the adult rat. *Stroke* 2004;35:239.

28. Besarab A, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto DM, Schwab SJ, Goodkin

DA. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *N Engl J Med* 1998;339:584-590.

29. Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, Boehm SM, Menne J, Haller H, Fliser D. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004;103:921-926.

30. Bahlmann FH, Song R, Boehm SM, Mengel M, von Wasielewski R, Lindschau C, Kirsch T, de Groot K, Laudeley R, Niemczyk E, Guler F, Menne J, Haller H, Fliser D. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. *Circulation* 2004;110:1006-1012.

31. Fiordaliso F, Chimenti S, Staszewsky L, Bai A, Carlo E, Cuccovillo I, Doni M, Mengozzi M, Tonelli R, Ghezzi P, Coleman T, Brines M, Cerami A, Latini R. A nonerythropoietic derivative of erythropoietin protects the myocardium from ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* 2005;102: 2046-2051.

32. Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M, Savino C, Bianchi M, Nielsen J, Gerwien J, Kallunki P, Larsen AK, Helboe L, Christensen S, Pedersen LO, Nielsen M, Torup L, Sager T, Sfacteria A, Erbayraktar S, Erbayraktar Z, Gokmen N, Yilmaz O, Cerami-Hand C, Xie QW, Coleman T, Cerami A, Brines M. Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 2004;305:239-242.

Discussion

Chapter 8

Erythropoietin: From Hematopoiesis to Cardioprotection

Peter van der Meer, Erik Lipsic, Wiek H. van Gilst,
Dirk J. van Veldhuisen

an editorial

Cardiovascular Drugs and Therapy 2005; 19: 7-8

Myocardial infarction accounts for increasing morbidity and mortality in western countries. Although the early reperfusion therapy significantly improves the clinical outcome, additional myocardial protection may be needed to limit reperfusion damage.

Various growth factors have been found to provide cell protection during reperfusion of the ischemic heart. Recently, hematopoetic hormone- erythropoietin (EPO) has been implicated in cardioprotection.

EPO is produced primarily in the kidney and its actions are mediated via a specific interaction with EPO-receptor (EPO-R). Major intracellular transduction pathways involved in EPO signalling include STAT-5, MAP and Akt kinases (1). Activation of these pathways is associated with cellular protection, predominantly due to inhibition of apoptosis. Presence of EPO in bone-marrow thus salvages the precursor cells from programmed death, enabling their proliferation and differentiation (2).

In recent years, expression of functional EPO-R has also been demonstrated in non-hematopoietic cells and organs including brain, kidney and cardiovascular tissue. These findings indicate that besides stimulating hematopoiesis, EPO may play a role as a pleiotropic survival and growth factor (3).

Many experimental studies have shown the protective effect of EPO in the brain. Systemic administration of EPO before or up to 6hours after focal brain ischemia reduced the infarct volume by 50-75% (4). In most models, inhibition of apoptosis was documented as the underlying mechanism of observed neuroprotection (5). In the first clinical, randomized, double-blind trial, EPO was given to patients with ischemic stroke presenting within 8 hours after onset of symptoms (6). In spite of the relatively small number of patients in this study, EPO administration in high-doses (entire dose 100.000 IU/ given in three days) proved to be both safe and beneficial. Patients randomized to the EPO group showed significant improvement in clinical outcome parameters and a trend towards smaller infarct sizes.

Recently, evidence is accumulating for a protective role of EPO in the heart. In a study performed by our group, presence of a functional EPO-R in rat heart was established (7). Furthermore, perfusion of isolated rat hearts with EPO during ischemia/ reperfusion (I/R) improved left ventricular function and limited cellular damage. Cai *et al* (8) showed that immediate protection against I/R provided by EPO is specifically mediated through an Akt-dependent pathway.

In vivo, Parsa *et al* evaluated the effects of EPO in a rabbit model of both I/R and permanent coronary occlusion. In both conditions, administration of EPO (5.000 IU/kg) led to infarct size reduction associated with inhibition of apoptosis (9).

However, two clinically important questions remained to be answered. What is the time window of opportunity for EPO administration during ischemic insult and what is the minimum dose that would still render cardioprotection. Our group has tried to answer the first question, showing the extension of the EPO cardioprotective effect beyond the start of reperfusion (10).

In this issue of Cardiovascular Drugs and Therapy, Hirata *et al*. (11) report that EPO treatment in a canine model of I/R reduced infarct size across a broad dose range from 100-1000 IU/kg. This study shows EPO treatment protects against I/R injury in the heart in a dose dependent manner. It does so, at least to some extent, by reducing apoptosis. The authors also demonstrate, for the first time *in vivo*, the crucial role the Akt-pathway plays in the observed effects. However, the proposed direct anti-arrhythmogenic role of EPO should be considered cautiously, as it may well have been directly the result of infarct size reduction.

Besides its acute cardioprotective effects, through inhibition of apoptosis, EPO may also influence formation of new vessels after ischemic injury. In vitro, stimulation of cultured endothelial cells with EPO results in proliferation and formation of vascular structures (12). Jaquet *et al*. compared the angiogenic properties of EPO with vascular endothelial growth factor (VEGF) on endothelial cells derived from human myocardial tissue. They found that both proteins exhibited equal angiogenic properties, indicating a possible pro-angiogenic effect of EPO (13). This was further studied in a rat model of stroke. EPO treatment, initiated 24 hours after occlusion of the middle cerebral artery, enhanced angiogenesis and improved neurological function, while it did not significantly influence infarct size (14). However, the mechanisms behind the effects of EPO on neovascularisation remain largely unknown. Very recently, Tepper *et al*. showed for the first time that newly formed vessels in hypoxic tissue were constructed entirely of bone marrow-derived endothelial progenitor cells (EPCs), known as vasculogenesis (15). Since EPO is a powerful stimulator of the mobilisation of endothelial progenitor cells from the bone marrow, it is tempting to speculate that the effects observed with EPO treatment might be related to the mobilisation of EPCs. However, the neovascularisation could also be related to sprouting of existing endothelial cells (i.e. angiogenesis). Further research will be needed to elucidate the effects of EPO on neovascularisation and the role of angiogenesis and/or vasculogenesis in this process.

The hematopoiesis stimulating function of EPO has been used for almost two decades in treating patients with renal anemia. Indications for EPO treatment have broadened in the last few years to include other forms of anemia, where EPO deficiency is not primarily causative. In clinical cardiology, EPO has already been used in anemic patients with chronic heart failure (CHF). Anemia is common in patients with CHF and related to an increased morbidity and mortality (16). Furthermore, not only anemia, but also elevated endogenous EPO levels are independently associated with an impaired outcome in CHF (17). Normalisation of hemoglobin levels in mildly anemic CHF patients has a positive effect on left ventricular ejection fraction (18) and peak VO_2 (19). In addition to correction of anemia, other non-hematopoietic effects of EPO, as described above, might play a role in the improvement observed in patients with CHF treated with EPO.

In summary, EPO is increasingly regarded a general tissue-protective agent. The new study of Hirata *et al*. in this issue of the journal provides additional evidence that EPO is cardioprotective even at low doses. Considering the high safety profile of EPO, future clinical studies in patients with acute coronary syndromes are a logical next step in broadening the indications for EPO therapy: from hematopoiesis stimulation to cardioprotection.

References

1. Ratajczak J, Majka M, Kijowski J *et al*. Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. Br J Haematol 2001;115:195-204.

2. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 1990;248:378-81.

3. Van der Meer P., Voors AA, Lipsic E, van Gilst WH, van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. Eur Heart J 2004;25:285-91.

4. Brines ML, Ghezzi P, Keenan S *et al*. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 2000;97:10526-31.

5. Cerami A, Brines M, Ghezzi P, Cerami C, Itri LM. Neuroprotective properties of epoetin alfa. Nephrol Dial Transplant 2002;17 Suppl 1:8-12.

6. Ehrenreich H, Hasselblatt M, Dembowski C *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 2002;8:495-505.

7. Van der Meer P, Lipsic E, Henning RH *et al*. Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury. Eur J Heart Fail 2004;6:853-9.

8. Cai Z, Semenza GL. Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. Circulation 2004;109:2050-3.

9. Parsa CJ, Matsumoto A, Kim J *et al*. A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest 2003;112:999-1007.

10. Lipsic E, Van der Meer P, Henning RH *et al*. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. J Cardiovasc Pharmacol 2004;44:473-9.

11. Hirata. Erythropoietin just before reperfusion reduces both lethal arrhythmia and infarct size via phos phatidylinositol-3 kinase-dependent pathway in canine hearts. Cardiovasc Drugs Ther 2004.

12. Ribatti D, Presta M, Vacca A *et al*. Human erythropoietin induces a pro-angiogenic phenotype in cul tured endothelial cells and stimulates neovascularization *in vivo*. Blood 1999;93:2627-36.

13. Jaquet K, Krause K, Tawakol-Khodai M, Geidel S, Kuck KH. Erythropoietin and VEGF exhibit equal angiogenic potential. Microvasc Res 2002;64:326-33.

14. Wang L, Zhang ZG, Wang Y, Zhang RL, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 2004;35:1732-7.

15. Tepper OM, Capla JM, Galiano RD *et al*. Adult vasculogenesis occurs through the in situ recruitment, proliferation and tubulization of circulating bone marrow-derived cells. Blood 2004.

16. Horwich TB, Fonarow GC, Hamilton MA, MacLellan WR, Borenstein J. Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. J Am Coll Cardiol 2002;39:1780-6.

17. Van der Meer P, Voors AA, Lipsic E, Smilde TDJ, van Gilst WH, van Veldhuisen DJ. Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure. J Am Coll Cardiol 2004;44:63-7.

18. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized con trolled study. J Am Coll Cardiol 2001;37:1775-80.

19. Mancini DM, Katz SD, Lang CC, LaManca J, Hudaihed A, Androne AS. Effect of erythropoietin on exer cise capacity in patients with moderate to severe chronic heart failure. Circulation 2003;107:294-9.

Chapter 9

Summary, conclusions and future directions

Summary

The purpose of the studies presented in this thesis was to investigate the origin of anemia in CHF patients and to study the influence of anemia and endogenous EPO levels on mortality in CHF (**part one**). Further, we investigated the ancillary properties of exogenous EPO on cardioprotection, besides the well-known hematopoietic effects of EPO (**part two**).

Etiology of anemia in CHF

It has been established in several studies that anemia is an independent prognostic marker for mortality in CHF patients. Although anemia is common in patients with CHF, the origin of anemia in CHF is mostly unknown. Several potential mechanisms may be involved (discussed in **chapter 2**).

Chronic renal failure (CRF) is likely to be an important contributory factor in the anemia observed in CHF patients. In many patients with end-stage renal disease, anemia is a common feature. In turn, CHF can cause CRF due to a decreased cardiac output and relative renal vasoconstriction, leading to chronic renal ischemia and subsequently inducing anemia. The links between CRF, CHF and anemia have been called the Cardio-Renal-Anemia syndrome([1]).

Since many patients with CHF have a normal or only slightly impaired renal function, other factors probably play a role in the presence of anemia in CHF. Hematinic deficiencies may be implicated to the anemia observed in CHF patients. There may be reduced uptake of iron, folate acid and vitamin B12, related with poor nutrition, malabsorption and cardiac cachexia([2]). Furthermore, the use of aspirin and oral anticoagulation can lead to microscopic amounts of gastrointestinal blood loss, contributing to the anemia. A recent study in the UK demonstrated that only a minority of CHF patients had renal impairment or underlying hematinic deficiencies([3]). This was confirmed by Witte *et al*, who showed that less than one third of the CHF patients were iron, folate acid or vitamin B12 deficient([4]).

Anemia can originate from increased plasma volume (hemodilution), due to salt and water retention in CHF patients([5]). Lower hemoglobin levels are also frequently observed in inflammatory conditions. It has been shown that patients with CHF express elevated levels of TNF-alpha, which in turn may reduce the hematopoietic proliferation. Experimental data support this hypothesis. The proliferative capacity of the progenitor cells in mice with heart failure was found reduced to approximately 50% of control([6]).

Treatment of CHF might induce anemia. It has been shown that intervention in the renin angiotensin system (RAS) may be related to reduced hematopoietic activity. A recent substudy of the Studies Of Left Ventricular Dysfunction (SOLVD), demonstrated convincingly that enalapril significantly increased the odds of developing anemia by 56% in patients with CHF([7]). To extend on these findings, a study in 98 CHF patients was performed to investigate the effects of ACE-inhibitor therapy on hematopoietic proliferation (**Chapter 3**). Almost 60% of the anemic patients had an unexplained anemia (normal hematinics and no renal failure). Serum of these anemic CHF patients inhibited in vitro the proliferation of bone marrow derived erythropoietic progenitor cells of healthy donors by 17%. Levels of the hematopoiesis inhibitor Ac-SDKP, which is almost exclusively degraded by ACE, were significantly higher in anemic CHF patients. The clear correlation between Ac-SDKP levels and proliferation of erythroid progenitor cells suggests an inhibitory role of Ac-SDKP on hematopoiesis in CHF patients, which may explain the observed anemia in patients treated with ACE-inhibitors.

Anemia, Endogenous EPO and Survival

Few studies have been performed on the role of endogenous EPO levels. The relation between endogenous EPO levels and hemoglobin levels may give further insight into the pathyphysiology of anemia in heart failure. It has been shown in populations with anemia of chronic disease, endogenous EPO levels are reported to be relatively low, compared to the degree of anemia([8;9]). In CHF it has been observed that endogenous EPO levels are elevated, proportional to the severity of symptoms([10;11]). In **chapter 4** the impact of endogenous EPO levels on hemoglobin and survival was studied. Patients with mild to severe CHF and control patients were included. Multivariate analysis showed that plasma EPO and hemoglobin levels were independent predictors of survival. These findings were recently confirmed by others in a slightly larger CHF population([12]). Furthermore, only a mild inverse correlation between EPO and hemoglobin levels could be observed in CHF patients, whereas the control group showed a clear significant inverse correlation, as expected. These findings may indicate a blunted EPO response relative to the hemoglobin levels. Further, it is tempting to speculate that due to the pro-inflammatory cytokines EPO resistance may occur in the bone marrow, requiring higher EPO levels to counterbalance the decreased sensitivity to EPO.

Pleiotropic effects of EPO

In response to lower oxygen levels, an upregulation of EPO occurs. EPO acts as a major regulator of erythropoiesis, by promoting the survival and proliferation of erythroid precursor cells, due to its anti-apoptotic properties. These anti-apoptotic effects resulted in extensive research in the anti-apoptotic properties of EPO in various models of neurological damage, including ischemic stroke and subarachnoid hemorrhage([11;13-15]). Since there are many similarities between ischemia in the brain and the heart, recently studies have been conducted to evaluate its possible effect in cardiac ischemia([16-18]). In **chapter 5** the presence and functionality of the EPO-receptor was studied by perfusing rat hearts with EPO or vehicle. Immunohistochemistry in this *ex vivo* model revealed the presence of the EPO-R on endothelial cells, fibroblasts and to a lesser extent cardiomyocytes. Furthermore, perfusion with EPO resulted in an increase in the phosphorylated MAP kinases p42/p44, whereas no differences in phosphorylated STAT5 could be observed. In a low-flow ischemia/reperfusion experimental setup EPO reduced the cellular damage by 56% during reperfusion and resulted in a significantly improved recovery of left ventricular pressure and coronary flow. Immunohistochemistry with staining against active-caspase 3 showed that EPO reduced apoptosis by approximately 15%.

EPO in experimental Heart Failure

These findings prompted further research of the non-hematopoietic effects of EPO in CHF. Another important pleiotropic effect of EPO is associated with its ability to stimulate endothelial cell proliferation([19]). As shown in chapter 5, the EPO-receptor is expressed on endothelial cells. These findings support the *in vivo* data regarding the effects of EPO on neovascularization. It has been shown in a rodent model of hind-limb ischemia, that EPO increases capillary density 1.6-fold([20]). In **chapter 6** we assessed the effects of EPO treatment in a rat model of post-myocardial infarction heart failure. This study demonstrated that EPO treatment in a rat model of heart failure improved cardiac function beyond its effect on infarct size reduction. It was shown that a single dose of EPO clearly improved cardiac performance, prolonged EPO treatment was associated with a further restoration of cardiac function. Mechanisms involved

in this process are most likely distinct from its acute cardioprotective effect. This is clearly demonstrated by the finding that EPO treatment, initiated three weeks after MI, although not reducing infarct size, significantly improved cardiac function, reflected by a 34% decrease in left ventricular end-diastolic pressure, and restoring N -ANP levels to sham values. Since the effect of EPO treatment in this group could not be explained by infarct size reduction, other properties of EPO should be considered to elucidate the observed beneficial effects of EPO in heart failure. In line with this finding, we found an increased capillary density and an increased capillary-to-myocyte ratio in the EPO treated groups, indicating actual capillary growth, which was related to myocardial function.
However, several questions remained unanswered. Since EPO treatment was also associated with an increased hematocrit value, the observed beneficial effects may, at least to some extend, be related to the increased oxygen-carrying capacity of blood. It has been shown that also lower doses of EPO resulted in vascular and kidney protection, without increasing hemoglobin levels([21]). Further it was unclear when the improvement in cardiac function occurred and whether EPO could mobilize endothelial progenitor cells (EPCs), which may be related to the neovascularization. To address these questions, we performed a similar study in which we included a second group treated with a low-dose EPO, besides the high-dose EPO (**chapter 7**). Echocardiography was used to subsequently measure the cardiac function during follow-up. In this study we found that both high and low-dose EPO treatment resulted in consolidation of left ventricular systolic function during follow-up, while in the control group left ventricular systolic function deteriorated over time. Although the infarct size was similar in all MI groups, both EPO-treated groups showed an improved cardiac function. In addition high-dose and low-dose EPO treatment increased the number of peripheral EPCs. Both EPO treatments resulted in a higher capillary density in the spared myocardium. The low-dose EPO treatment did not increase the hematocrit, and therefore the observed beneficial effects are entirely related to the non-hematopoietic effects of EPO.

Future directions

Etiology of anemia in CHF

In part 1 of this thesis the etiology of anemia in CHF is described. The finding that Ac-SDKP may explain the occurrence of anemia in CHF provides a new link between hematopoiesis and the RAS. Further evidence for an important role of Ac-SDKP in anemia was provided in anemic hemodialysis patients. They found that patients on hemodialysis with high Ac-SDKP levels required higher weekly EPO doses to prevent development of anemia([22]). Therefore, it is interesting to speculate that in patients with CHF treated with ACE-inhibitors anemia will only develop when lower EPO levels exists in conjunction with higher Ac-SDKP levels. It follows that under these circumstances, the inhibitory effect of Ac-SDKP exceeds the proliferative properties of EPO. Furthermore, one may hypothesize that the inhibitory effects of ACE-inhibitors on hematopoiesis can be prevented by the use of angiotensin receptor blockers in CHF patients. However, since angiotensin II stimulates the proliferation of erythroid-progenitor cells, treatment with angiotensin receptor blockers might also lower the hematopoietic activity ([23]). Theoretically, the combination of ACE-inhibitors and angiotensin receptor blockers may be deleterious for the hematopoietic proliferation activity. Clearly, further studies are needed to address these questions.

In addition, it remains speculative whether correction of anemia in CHF patients may lead to a reduced morbidity and mortality. Several small-scale trials showed beneficial effects of EPO treatment on exercise capacity, left ventricular function and hospital admission. However, it must be noted that these studies were not performed in a double-blinded manner, which may influence outcome([24;25]). It also needs to be elucidated what the optimal hemoglobin target would be in anemic CHF patients, whether the hemoglobin targets used in renal anemia can be extrapolated to the CHF population and when EPO treatment should be initiated. In addition, multi-center trials in CHF patients should be performed, to study the impact of EPO treatment on morbidity and mortality. At the present, treatment of anemia in patients with CHF is not the standard of care and EPO treatment should only be assessed in an investigational setting, not withstanding its important role in the management of renal anemia and the promising results of small scale (pre)-clinical trials.

Pleiotropic effect of EPO

Besides the direct hematopoietic effects of EPO, it has been shown that EPO possesses several non hematopoietic effects, including neovascularization. However, it still needs to be elucidated whether the neovascularization is mainly related to proliferation of in situ endothelial cells (i.e. angiogenesis) or is associated with vasculogenesis. In a recent paper it has been shown that newly formed vessels in hypoxic tissue were constructed entirely of bone marrow-derived endothelial progenitor cells (EPCs), a process known as vasculogenesis([26]). Since EPO is a powerful stimulator of the mobilization of endothelial progenitor cells from the bone marrow in CHF, as shown in chapter 7, it is tempting to speculate that the effects observed with EPO treatment might be related to the mobilization of EPCs. On the other hand, neovascularization could also be related to sprouting of existing endothelial cells. It has been shown that EPO is able to stimulate the proliferation of endothelial cells, in vitro and *in vivo*. To discern between vasculogenesis and angiogenesis transgenic studies would be needed in which wild-type rats will be lethally irradiated and bone marrow will be substituted from transgenic rats. By co-staining for an endothelial marker and a reporter protein, endothelial cells derived from the bone marrow can be detected. These studies will further increase our knowledge of the effects of EPO on neovascularization in CHF.

There is limited evidence for pleitropic effects of EPO in human studies. One of the first studies, assessing the effects of EPO on infarct size reduction was performed by Ehrenreich and colleagues. In a double blind randomised proof-of-concept trial they investigated the safety and efficacy of EPO in stroke patients([27]). Forty patients received either EPO or saline daily for 3 days after stroke. The investigators found an improvement in clinical outcome and a trend towards reduction in infarct size, assessed by MRI-scan, in the EPO treated patients. Recently, we performed a similar safety study in patients with acute myocardial infarction (manuscript in preparation). Twenty-two patients were randomized to EPO (darbopoetin 300μg) or placebo. No adverse events were recorded during the 30-day follow up. In the EPO treated patients, only a non-significant increase in hemoglobin levels could be observed. In addition, EPO treatment was associated with elevated levels of endothelial progenitor cells. Larger scale clinical trials, assessing the effects of EPO on infarcts size and left ventricular function are warranted. The effects of EPO on endothelial progenitors might be of benefit in the treatment of CHF patients. Therefore further investigation is needed to provide more insight into the effects of EPO in CHF. Positron Emission Tomography (PET) might be a useful tool to study the effects of EPO on cardiac metabolism and myocardial blood flow and

its relation to endothelial progenitor cells.

Recent studies in brain and heart focussed on EPO analogues without hematopoietic effects. One of these compounds is carbamylated EPO (C-EPO). Through carbamylation, which slightly modifies the lysine residues in EPO, a non-hematopoietic EPO is created which does not possess erythropoietic effects, but still renders tissue protection. *In vivo* studies in rodents showed that even high doses of C-EPO for longer periods of time did not result in an increase in hemoglobin values([28]). However, it has been shown that C-EPO inhibits apoptosis, decreases infarct size and subsequently improves cardiac function in rats subjected to myocardial infarction([29]). The development of these EPO derivates might be of potential benefit in the treatment of myocardial infarction and CHF in patients with normal hemoglobin levels. The discovery of these EPO analogues and the effects beneficial of low-dose EPO may shift the role of EPO from hematopoiesis to cardioprotection.

References

1. Silverberg DS, Wexler D, Iaina A. The importance of anemia and its correction in the management of severe congestive heart failure. Eur J Heart Fail. 2002;4:681-686.

2. Anker SD, Sharma R. The syndrome of cardiac cachexia. Int J Cardiol. 2002;85:51-66.

3. Cromie N, Lee C, Struthers AD. Anaemia in chronic heart failure: what is its frequency in the UK and its underlying causes? Heart. 2002;87:377-378.

4. Witte KK, Desilva R, Chattopadhyay S *et al*. Are hematinic deficiencies the cause of anemia in chronic heart failure? Am Heart J. 2004;147:924-930.

5. Androne AS, Katz SD, Lund L *et al*. Hemodilution is common in patients with advanced heart failure. Circulation. 2003;107:226-229.

6. Iversen PO, Woldbaek PR, Tonnessen T *et al*. Decreased hematopoiesis in bone marrow of mice with congestive heart failure. Am J Physiol Regul Integr Comp Physiol. 2002;282:R166-R172.

7. Ishani A, Weinhandl E, Zhao Z *et al*. Angiotensin-converting enzyme inhibitor as a risk factor for the development of anemia, and the impact of incident anemia on mortality in patients with left ventricular dysfunction. J Am Coll Cardiol. 2005;45:391-399.

8. Means RT, Jr., Krantz SB. Progress in understanding the pathogenesis of the anemia of chronic disease. Blood. 1992;80:1639-1647.

9. Miller CB, Jones RJ, Piantadosi S *et al*. Decreased erythropoietin response in patients with the anemia of cancer. N Engl J Med. 1990;322:1689-1692.

10. Chatterjee B, Nydegger UE, Mohacsi P. Serum erythropoietin in heart failure patients treated with ACE-inhibitors or AT(1) antagonists. Eur J Heart Fail. 2000;2:393-398.

11. Volpe M, Tritto C, Testa U *et al*. Blood levels of erythropoietin in congestive heart failure and correla tion with clinical, hemodynamic, and hormonal profiles. Am J Cardiol. 1994;74:468-473.

12. George J, Patal S, Wexler D *et al*. Circulating erythropoietin levels and prognosis in patients with congestive heart failure: comparison with neurohormonal and inflammatory markers. Arch Intern Med. 2005;165:1304-1309.

13. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature. 2001;412:641-647.

14. Grasso G. Neuroprotective effect of recombinant human erythropoietin in experimental subarachnoid hemorrhage. J Neurosurg Sci. 2001;45:7-14.

15. Siren AL, Fratelli M, Brines M *et al*. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci U S A. 2001;98:4044-4049.

16. Calvillo L, Latini R, Kajstura J *et al*. Recombinant human erythropoietin protects the myocardium from ischemia- reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci U S A. 2003;100:4802-4806.

17. Moon C, Krawczyk M, Ahn D *et al*. Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. Proc Natl Acad Sci U S A. 2003;100: 11612-11617.

18. Parsa CJ, Matsumoto A, Kim J *et al*. A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest. 2003;112:999-1007.

19. Ribatti D, Presta M, Vacca A *et al*. Human erythropoietin induces a pro-angiogenic phenotype in cul tured endothelial cells and stimulates neovascularization *in vivo*. Blood. 1999;93:2627-2636.

20. Heeschen C, Aicher A, Lehmann R *et al*. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood. 2003;102:1340-1346.

21. Bahlmann FH, Song R, Boehm SM *et al*. Low-dose therapy with the long-acting erythropoietin ana logue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. Circulation. 2004;110:1006-1012.

22. Le Meur Y, Lorgeot V, Comte L *et al*. Plasma levels and metabolism of AcSDKP in patients with chronic renal failure: relationship with erythropoietin requirements. Am J Kidney Dis. 2001;38:510-517.

23. Mrug M, Stopka T, Julian BA *et al*. Angiotensin II stimulates proliferation of normal early erythroid progenitors. J Clin Invest. 1997;100:2310-2314.

24. Mancini DM, Katz SD, Lang CC *et al*. Effect of erythropoietin on exercise capacity in patients with moderate to severe chronic heart failure. Circulation. 2003;107:294-299.

25. Silverberg DS, Wexler D, Sheps D *et al*. The effect of correction of mild anemia in severe, resistant congestive heart failure using subcutaneous erythropoietin and intravenous iron: a randomized con trolled study. J Am Coll Cardiol. 2001;37:1775-1780.

26. Tepper OM, Capla JM, Galiano RD *et al*. Adult vasculogenesis occurs through the in situ recruitment, proliferation and tubulization of circulating bone marrow-derived cells. Blood. 2004;105:1068-1077.

27. Ehrenreich H, Hasselblatt M, Dembowski C *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med. 2002;8:495-505.

28. Leist M, Ghezzi P, Grasso G *et al*. Derivatives of erythropoietin that are tissue protective but not eryth ropoietic. Science. 2004;305:239-242.

29. Fiordaliso F, Chimenti S, Staszewsky L *et al*. A nonerythropoietic derivative of erythropoietin protects the myocardium from ischemia-reperfusion injury. Proc Natl Acad Sci U S A. 2005;102:2046-2051.

Nederlandse Samenvatting

Nederlandse Samenvatting

Hartfalen is een complex van klachten en verschijnselen ten gevolge van een tekort schietende pompfunctie van het hart. Patiënten met hartfalen hebben een hoog risico om te komen overlijden. In een recente studie is aangetoond dat het risico om tijdens het leven hartfalen te ontwikkelen 1 op 5 is voor zowel mannen als vrouwen. De etiologie van hartfalen is divers, maar de belangrijkste oorzaak is een doorgemaakt hartinfarct. Andere oorzaken voor het ontstaan van hartfalen zijn: hypertensie, hartklep afwijkingen, myocarditis en cardiomyopathie. Verschillende factoren zijn betrokken bij de prognose van hartfalen. Recent is bekend geworden dat bloedarmoede (anemie) een onafhankelijke voorspeller is voor mortaliteit in patiënten met hartfalen. De incidentie van anemie hangt van verschillende factoren af. Een van de belangrijkste determinanten is de ernst van het hartfalen. Naar mate de ernst toeneemt, neemt ook de incidentie van anemie toe. Hoewel verschillende onderzoeken de prognostische waarde van anemia voor het voorspellen van mortaliteit beschrijven, is over de oorzaak van anemia in hartfalen weinig bekend.
In **hoofdstuk 3** van dit proefschrift tonen we aan dat in 60% van de patiënten met hartfalen en anemie geen verklaring voor de bloedarmoede kan worden gevonden. Deze patiënten hebben geen nierfunctie stoornissen of ijzer- en vitaminedeficiënties. Het serum van deze anemische patiënten met hartfalen remt de proliferatie van erythroïde voorloper cellen. Dit in tegenstelling tot niet anemische hartfalen patiënten en gezonde controle personen. De oorzaak van de remming komt door hogere spiegels van de hematopoëse remmer, N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), in het serum van anemische hartfalers. Ac-SDKP is een tetrapeptide dat uitsluitend door ACE wordt afgebroken. Daarnaast werd een duidelijke correlatie gevonden tussen de Ac-SDKP spiegels en de proliferatie van hematopoëtische voorloper cellen. Deze bevindingen koppelen het renine angiotensine systeem aan hematopoëse en verklaren deels het optreden van bloedarmoede bij patiënten met hartfalen.
Er is weinig onderzoek gedaan naar de effecten van endogeen erythropoëtine (EPO) in patiënten met hartfalen. De relatie tussen hemoglobin en endogeen EPO kan meer inzicht geven in de pathofysiologie van anemie in patienten met hartfalen. In patienten met anemie door chronische ziekte is aangetoond dat EPO spiegels lager zijn dan men verwacht in relatie tot de ernst van de anemie. Dit zou een relatieve deficiëntie van EPO betekenen. In patiënten met hartfalen is in een tweetal onderzoeken aangetoond dat EPO spiegels stijgen als de ernst van het hartfalen toeneemt. In **hoofdstuk 4** werd gekeken naar het effect van endogene EPO en hemoglobine spiegels op mortaliteit in patienten met hartfalen. Multivariate analyse liet zien dat endogeen EPO en hemoglobine beide onafhankelijke voorspellers zijn voor mortaliteit in patiënten met mild tot ernstig hartfalen. Daarnaast vonden we in hartfalen patiënten een zwakke correlatie tussen hemoglobin en EPO, terwijl in de controle groep (zonder hartfalen) een sterke correlatie tussen hemoglobine en EPO werd gezien. Deze bevindingen vormen een aanwijzing dat de EPO response in hartfalen patiënten verminderd is, in relatie tot de hemoglobine waarden. Een mogelijke verklaring hiervoor kan zijn dat pro-inflammatoire cytokines zorgen voor een EPO resistentie in het beenmerg. Hierdoor zijn hogere EPO spiegels nodig om de hematopoëse voldoende te stimuleren.

Naast de hematopoëtische effecten van EPO zijn er ook non-hematopoëtische effecten van EPO bekend. Een belangrijke eigenschap van EPO is het anti-apoptotische effect. In een experimenteel herseninfarct model is aangetoond dat EPO toediening zorgt voor een reductie van

de infarctgrootte. Deze afname lijkt voor een belangrijk gedeelte te komen doordat EPO de apoptose remt. In **hoofdstuk 5** laten we zien dat er een functionele EPO-receptor aanwezig is in het rattenhart. De EPO receptor komt voornamelijk tot expressie op endotheel cellen en fibroblasten. Daarnaast werd in een 'low-flow' ischemie/reperfusie experiment aangetoond dat EPO behandeling leidde tot een verbeterde hartfunctie en coronair doorstroming. Deze positieve effecten kunnen deels worden verklaard doordat EPO het aantal apoptotische cellen in het hart met 15% verminderde.

Deze bevindingen hebben geleid tot verder onderzoek naar de non-hematopoëtische effecten van EPO in een experimenteel model voor hartfalen. Het is al eerder aangetoond dat EPO kan leiden tot proliferatie van gekweekte endotheel cellen. In een *in vivo* model van ischemie van de achterpoot van een rat, werd gezien dat EPO zorgde voor een toename in het aantal capillairen. In **hoofdstuk 6** hebben we de effecten van EPO bestudeerd in een rattenmodel voor post-infarct hartfalen. In deze studie werd aangetoond dat EPO behandeling, gestart drie weken na het myocard infarct, niet leidde tot een infarctgrootte reductie, maar wel een significante verbetering van de hartfunctie gaf. EPO resulteerde in een significante verhoging van het aantal capillairen per vierkante millimeter en zorgde voor een toename van het aantal capillairen per cardiomyocyt. Deze bevindingen laten zien dat de verbetering van de hartfunctie mogelijk gerelateerd is aan neovascularisatie.

Toch bleven er vele vragen onbeantwoord bij deze studie. EPO behandeling was namelijk ook geassocieerd met een significante stijging van de hematocriet. Een deel van de positieve effecten van EPO kunnen mogelijk verklaard worden door de verhoogde zuurstoftransportcapaciteit van het bloed. In eerdere studies naar de effecten van EPO op nierprotectie werd al aangetoond dat lagere EPO dosering de nieren en de bloedvaten beschermden, zonder dat dit leidde tot een stijging van de hematocrit. Verder was het onbekend wanneer de verbetering in hartfunctie ontstond en of de verhoogde capillaire dichtheid gerelateerd was aan het vermogen van EPO om endotheel voorloper cellen te kunnen mobiliseren.Om deze onderzoeksvragen te kunnen beantwoorden hebben in **hoofdstuk 7** naast de hoge dosis EPO ook een groep met een lage EPO dosering bestudeerd. Echocardiografie werd toegepast om sequentieel de hartfunctie te kunnen meten. In deze studie vonden we dat zowel de hoge als de lage dosering leidde tot een consolidatie van de hartfunctie, terwijl de hartfunctie in de onbehandelde controle groep in de tijd verslechterde. Hoewel infarctgrootte in alle groepen gelijk was, lieten beide EPO behandelde groepen een verbeterde hartfunctie zien. Daarnaast vonden we dat beide doseringen resulteerden in een toename van de circulerende endotheel voorloper cellen en leidden tot een hogere capillaire dichtheid. De lage EPO dosering zorgde niet tot een toename in de hematocriet. Hieruit kan worden geconcludeerd dat de effecten van de lage EPO dosering volledig zijn toe te schrijven aan de non-hematopoëtische eigenschappen van EPO.

Discussie

Oorzaak van anemie in chronisch hartfalen

In het eerste gedeelte van dit proefschrift wordt de etiologie van anemie in hartfalen patiënten bestudeerd. Serum van anemische hartfalen patiënten, met hogere Ac-SDKP spiegels, remde de proliferatie van hematopoëtische voorlopercellen. Hierin wordt voor het eerst de link tussen hematopoëse en het renine angiotensine systeem beschreven in patiënten met hartfalen. Dat Ac-SDKP een belangrijke rol speelt in het ontstaan van anemie werd ook aangetoond in dialyse patiënten. Hemodialyse patiënten met hogere Ac-SDKP spiegels hadden een hogere dosering van exogeen EPO nodig om de streefwaarden van het hemoglobine te bereiken. Mogelijk dat ACE-remmer gebruik bij patiënten met hartfalen pas tot anemie zal leiden, als er ook relatief lage endogene EPO spiegels aanwezig zijn. In deze conditie is het remmende effect van Ac-SDKP groter dan het stimulerende effect van EPO. Daarnaast zou het kunnen dat de negatieve effecten van ACE-remmers op de hematopoëse kunnen worden voorkomen door het gebruik van angiotensine receptor blokkers, die de ACE-activiteit niet verlagen en daardoor geen effect van Ac-SDKP spiegels hebben. Aan de andere kant is ook bekend dat angiotensine II leidt tot stimulering van de proliferatie van hematopoëtische voorlopercellen. Het blokkeren van de angiotensine II receptor kan mogelijkerwijs de proliferatie remmen. Theoretisch zou men kunnen stellen dat de combinatie van ACE-remmers en angiotensine receptor blokkers de proliferatie van hematopoëtische voorlopercellen het meest zou remmen. Meer onderzoek naar de effecten van beide geneesmiddelen op het hematopoëtische systeem zijn nodig om deze vragen te kunnen beantwoorden.

Verder is het op dit moment niet duidelijk of het corrigeren van de anemie in hartfalen patiënten ook daadwerkelijk leidt tot een reductie in morbiditeit en mortaliteit. Enkele kleine onderzoeken lieten wel een positief effect zien op uithoudingsvermogen, linker ventrikel functie en het aantal ziekenhuis opnames. Deze onderzoeken werden echter niet dubbelblind gerandomiseerd uitgevoerd, hetgeen een effect op de uitkomst kan hebben. Vooralsnog is het wachten op een groot dubbel blind 'multi-center' onderzoek, om het effect van EPO op mortaliteit en morbiditeit te analyseren. Momenteel behoort de EPO behandeling van anemische hartfalen patiënten niet tot de standaard zorg, hetgeen geen afbreuk doet aan de belangrijke rol van EPO in de therapie van renale anemie en de positieve resultaten van kleinschalige (pre)klinische studies in hartfalen.

Pleiotrope effecten van EPO

Naast de hematopoëtische effecten van EPO, bezit EPO ook non-hematopoëtische eigenschappen zoals neovascularisatie. Op dit moment is niet bekend of het effect van EPO op endotheel cellen leidt tot lokale proliferatie (angiogenese) of dat EPO zorgt voor vasculogenese. Recent onderzoek heeft laten zien dat nieuw gevormde bloedvaatjes in hypoxisch weefsel volledig bestonden uit endotheel cellen afkomstig uit het beenmerg. Dit proces is bekend onder de naam vasculogenese. EPO toediening leidt tot mobilisatie van endotheel voorloper cellen uit het beenmerg, zoals in hoofdstuk zeven is aangetoond. Daarom zouden de effecten van EPO op neovascularisatie gerelateerd kunnen zijn aan de mobilisatie van endotheel voorlopercellen uit het beenmerg. Aan de andere kant is het aangetoond dat EPO de proliferatie van endotheel cellen stimuleert. Om het effect van EPO op vasculogenese te kunnen onderscheiden van angiogenese zijn transgene studies noodzakelijk. Hierin worden non-transgene ratten bestraald en behandeld met het beenmerg van transgene ratten. Door

dubbel te kleuren voor markers van endotheel cellen én het reporter-eiwit kunnen endotheel cellen afkomstig uit het beenmerg worden gedetecteerd. Deze studies zullen meer inzicht geven in de effecten van EPO op neovascularisatie.

Over de pleiotrope effecten van EPO in humane studies is weinig bekend. De effecten van EPO op reductie van infarct grootte werden bestudeerd in patiënten met een herseninfarct. Ehrenreich en collega's lieten in een dubbel blind gerandomiseerde studie zien dat EPO toediening na een herseninfarct leidde tot een verbeterd functioneren en een trend tot een kleiner infarct. Recent hebben we in een kleine veiligheidsstudie de effecten van EPO bestudeerd in patiënten met een acuut hartinfarct. Tweeëntwintig patiënten werden gerandomiseerd naar EPO (darbepoetin 300 μg) of placebo. Er werden geen 'adverse events' gezien tijdens de 30 dagen follow-up. De EPO behandelde patiënten lieten een non-significante stijging zien in hemoglobine waarden. Daarnaast werd er een significante stijging van het aantal endotheel voorloper cellen gezien. Grotere klinische studies zijn nodig om deze pleiotrope effecten van EPO te onderzoeken. Met behulp van een Positron Emission Tomography (PET) scan zouden de effecten van EPO op het cardiale metabolisme en myocardiale bloedstroom kunnen worden onderzocht en de relatie met endotheel voorloper cellen.

Andere interessante ontwikkelingen op het gebied van pleiotrope effecten van EPO worden gezien op het vlak van EPO-analogen zonder hematopoëtische effecten. Een van deze middelen is gecarbamyliseerd EPO (C-EPO). Carbamylisatie verandert de structuur van het EPO molecuul, waardoor het geen hematopoëtische effecten meer heeft, maar nog wel weefsel protectie geeft. *In vivo* is in knaagdieren aangetoond dat hoge C-EPO doses gedurende langere tijd geen verhoging van het hematocriet gaf. Toch remde C-EPO apoptose, reduceerde het de infarctgrootte en leidde uiteindelijk tot een verbeterde hartfunctie. De ontwikkeling van deze EPO-analogen kunnen mogelijk een rol spelen in de behandeling van het myocard infarct en hartfalen in patiënten met normale hemoglobine waarden. De ontdekking van EPO-analogen zonder hematopoëtische eigenschappen en het effect van lage doseringen EPO verplaatst de rol van EPO van hematopoëse naar cardiale bescherming.

Dankwoord

Zoals ieder dankwoord eigenlijk begint: promoveren doe je niet alleen en dat is natuurlijk ook zo. Veel personen zijn betrokken geweest bij het totstandkomen van dit proefschrift, hiervoor mijn grote dank. Een aantal van hen wil ik graag persoonlijk bedanken.

Ten eerste mijn eerste promotor prof. dr. D.J. van Veldhuisen. Beste Dirk-Jan, vanaf het eerste moment, toen ik nog co-assistent was, heb je me enthousiast gemaakt voor het onderzoek naar erythropoëtine en anemie. Ik wil je bedanken voor de kansen die je me hebt gegeven om veel (inter)nationale ervaring op te doen. Ik waardeer je efficiënte manier van werken en je praktische instelling om het onderzoek te managen. Verder wil ik je bedanken voor de mogelijkheid om mij te kunnen specialiseren tot cardioloog.

Ten tweede wil ik mijn tweede promotor prof. dr. W.H. van Gilst bedanken. Beste Wiek, toen ik je de eerste keer ontmoette, moest ik meteen aan jouw colleges over het renine angiotensine systeem denken. Dat uiteindelijk zelfs een proefschrift over anemie en erythropoëtine nog over ACE zou gaan, had ik toen niet gedacht. Jouw idee om ACE-remmers en hematopoëse te linken door Ac-SDKP, resulteerde in een mooie publicatie. Ik waardeer je betrokkenheid bij mijn promotie zeer. Jouw scherpe analyses, je humor en laagdrempeligheid maken je tot een prettig persoon om mee te werken. Mijn dank hiervoor.

Verder wil ik mijn beide copromotores Dr. A.A. Voors en Dr. R.G. Schoemaker bedanken. Beste Adriaan, ons eerste contact hadden we toen jij nog assistent in Nieuwegein was en het duidelijk werd dat je naar Groningen zou komen. De afgelopen 3 jaar ben je bij het EPO-project betrokken geweest, hiervoor mijn dank. Hoewel ik zelf niet zo'n auto-freak ben, moet ik toch toegeven dat het rijden van Groningen naar Delft in jouw Jaguar een prima alternatief was voor de trein of Renault. Beste Regien, bedankt voor je inzet voor, in het bijzonder, het experimentele gedeelte van het onderzoek. Jouw ervaringen met het hartfalen model in Rotterdam waren erg nuttig.

De leden van de beoordelingscommissie, Prof. dr. T.J. Rabelink, Prof. dr. E.G. de Vries en Prof. dr. F. Zijlstra, wil ik bedanken voor de bereidheid om mijn proefschrift kritisch te beoordelen.

Daarnaast wil dr. R.H. Henning bedanken. Beste Rob, ondanks dat het EPO onderzoek (tot nu toe) weinig gericht is geweest op het cellulaire mechanisme, ben je vanaf het begin betrokken. Ik waardeer je heldere analyses en de levendige discussies.

Zonder de ondersteuning van velen op de Klinische Farmacologie was dit proefschrift er niet gekomen. Hiervoor wil ik Egbert, Alex, Jacko, Kristien, Marry, Cecile, Wessel, Bianca, Maaike en Liza bedanken voor hun hulp. Verder wil ik Azuwerus van Buiten bedanken voor zijn bereidheid om soms 's avonds mee te helpen als de experimenten weer eens uitliepen.

Johan Koster en Gideon du Marchie Servaas wil ik bedanken voor hun inzet om bij nacht en ontij echocardiogrammen te maken bij de hartfalen ratten.

Graag wil ik ook Alma en Olga, de twee secretaresses van de cardiologie bedanken voor de

hulp die jullie hebben geboden tijdens de promotie, maar ook zeker tijdens de laatste loodjes van het proefschrift nu ik in Deventer zit. Mijn dank hiervoor. Daarnaast wil ik Ardy, Alexandra en Ellen, secretaresses bij de Klinische Farmacologie bedanken

'Van je collega's moet je het maar hebben'. Het had stelling 11 kunnen zijn en ik onderschrijf hem helemaal. De sfeer onder elkaar maakt het werken prettig. Hiervoor wil ik alle leden en ex-leden van de Greenhouse bedanken: Pim, Tom, Folkert, Wim, Daan, de 4x'M' "AF is a benign disease" Michiel, Marcella, Martin, Meint, Vincent, Eduard, Tone, Jessica, Christiane en Pieter.

Tevens wil ik de collega's van de Klinische Farmacologie bedanken: Peter, Maria, Els, Annemarieke, Cheng, Ying, Mirjam, Willemijn, Bas, Bernadette en Bart (bier drinken aan de bar in een Slowaakse trein is zo gek nog niet). Verder wil ik Jan Veerbeek en Jurjen Zandstra bedanken voor hun werk voor het EPO-project.

Als arts-onderzoekers was er altijd een goede samenwerking met de cardio-research. Allereerst wil ik Greetje bedanken voor de hulp bij alle trials en natuurlijk de gezelligheid. De vele internationale bijeenkomsten waren altijd een succes. Ook Peter wil ik bedanken voor zijn onuitputtelijke geduld met alle handtekening op de CRF's en de vele meetings met vele single malt whiskey's. Verder wil ik Anja, Zaza, Trynke en Margriet bedanken.

Verder wil ik mijn beide paranimfen bedanken voor hun hulp en steun. Ten eerste Erik Lipsic, mijn directe collega op het EPO-project. Beste Erik, vanaf het allereerste moment waren we beiden betrokken bij hetzelfde onderzoek. Ik waardeer je relativeringsvermogen en de rust die je uitstraalt. Ik ben er trots op dat we echte vrienden zijn geworden en ik ben blij dat je mijn paranimf wilt zijn. Er is trouwens niks mis met Deventer.
Ten tweede wil ik Jeroen Koning bedanken. Beste Jeroen, toen onze beide vriendinnen nog in Amsterdam woonden, wisten wij al dat er niets boven Groningen ging. Onze gezamenlijke interesses in goed eten, wijn en wandelen zijn altijd een goede basis voor vakanties en weekendjes weg. Ik hoop dat we dit in de toekomst nog vaak met jou en Ada ondernemen.

Tot slot wil ik mijn ouders bedanken. Lieve Heit en Mem, bedankt voor alle onvoorwaardelijke steun die jullie Anna-Rixt en mij door de jaren hebben gegeven. Jullie nuchtere kijk op het leven en interesse in ons, al moesten jullie ons opzoeken in Nicaragua, waardeer ik heel erg.

Lieve Margreet, hoe zou ik jou hier nu in enkele zinnen kunnen bedanken voor wat je door de jaren heen voor mij hebt betekend. Jouw hulp en geduld tijdens alle drukte rondom mijn proefschrift waardeer ik zeer. Ik ben ervan overtuigd dat we er nog vaak op uit trekken in binnen- en buitenland en een goede tijd tegemoet gaan in Deventer.

Bibliography

1. **Van der Meer P**, Ulrich AM, Gonzalez-Scarano F, Lavi E. Immunohistochemical analysis of CCR2, CCR3, CCR5, and CXCR4 in the human brain: potential mechanisms for HIV dementia. Exp Mol Pathol 2000; 69:192-201

2. **Van der Meer P**,Goldberg SH, Fung KM, Sharer LR, Gonzalez-Scarano F, Lavi E. Expression pattern of CXCR3, CXCR4, and CCR3 chemokine receptors in the developing human brain. J Neuropathol Exp Neurol 2001; 60:25-32

3. Goldberg SH, **Van der Meer P**, Hesselgesser J, Jaffer S, Kolson DL, Albright AV, Gonzalez-Scarano F, Lavi E. CXCR3 expression in human central nervous system diseases. Neuropathol.Appl.Neurobiol 2001; 27:127-138.

4. **Van der Meer P**, Quant-Durán C. Diagnostic image. A Nicaraguan man with an epileptic seizure: Neu rocysticercosis. Ned Tijdschr Geneeskd. 2004;148:221

5. **Van der Meer P**, Van Gilst WH, Van Veldhuisen DJ Anemia and congestive heart failure. Circulation 2003;108:e41-42 *[letter]*

6. **Van der Meer P**, Voors AA, Lipsic E, Van Gilst WH, Van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. Eur Heart J 2004; 25:285-291.

7. **Van der Meer P,** Voors AA, Lipsic E, Smilde TD, Van Gilst WH, Van Veldhuisen DJ. Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure. J Am Coll Cardiol 2004; 44:63-67.

8. **Van der Meer P**, Lipsic E, Henning RH, De Boer RA, Suurmeijer AJ, Van Veldhuisen DJ, Van Gilst WH. Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury. Eur J Heart Fail 2004; 6:853-859.

9. Lipsic E, **Van der Meer P**, Henning RH, Suurmeijer AJ, Boddeus KM, Van Veldhuisen DJ, Van Gilst WH, Schoemaker RG. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. J Cardiovasc Pharmacol 2004; 44:473-479.

10. **Van der Meer P**, De Boer RA, White HL, Van der Steege G, Hall AS, Voors AA, Van Veldhuisen DJ. The VEGF +405 CC Promoter Polymorphism is Associated With an Impaired Prognosis in Patients With Chronic Heart Failure: A MERIT-HF Substudy. J Card Fail 2005; 11:279-284.

11. Lipsic E., Van der Horst IC, Voors AA, **Van der Meer P**, Nijsten MW, Van Gilst WH, Van Veldhuisen DJ, Zijlstra F. Hemoglobin levels and 30-day mortality in patients after myocardial infarction. Int.J.Cardiol 2005; 100:289-292.

12. **Van der meer P**, Lipsic E, Van Veldhuisen DJ, Van Gilst WH. Erythropoietin: from hematopoiesis to cardioprotection. Cardiovasc Drugs Ther 2005;19:7-8 *[editorial]*.

13. **Van der Meer P**, Lipsic E, Henning RH, Boddeus K, Van der Velden J, Voors AA, Van Veldhuisen DJ, Van Gilst WH, Schoemaker RG. Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction. J.Am.Coll.Cardiol. 2005; 46:125-133.

14. **Van der Meer P,** Lipsic E, Westenbrink BD, Van de Wal RM, Schoemaker RG, Vellenga E, Van Veldhuisen DJ, Voors AA, Van Gilst WH. Levels of hematopoiesis inhibitor N-acetyl-seryl-aspartyl-lysyl-proline partially explain the occurrence of anemia in heart failure. Circulation 2005; 112: 1743-1747.